DIARRHEAL DISEASES IN RURAL BANGLADESH: SPATIAL-TEMPORAL PATTERNS, RISK FACTORS AND PATHOGEN DETECTION

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ABSTRACT

Jianyong Wu: Diarrheal Diseases in rural Bangladesh: spatial-temporal patterns, risk factors and pathogen detection
(Under the direction of Michael Emch and Jill Stewart)

Diarrheal diseases are still a leading cause of child mortality in less developed countries. In the past three decades, in an effort to reduce the transmission of diarrheal diseases, millions of tubewells have been installed as a way to provide safe drinking water in Bangladesh. However, this effort may have been counterproductive since widespread arsenic contamination has been found in groundwater. Thus, there is a reason to rethink the use of tubewells and to assess risk factors related to diarrheal disease in Bangladesh. This study primarily focused on 142 villages of Matlab, a rural area in Bangladesh, using datasets collected through a local health surveillance system to explore the spatiotemporal patterns of diarrheal disease and its relevant risk factors. First, a geographic information system (GIS) and spatial statistics were used to illustrate the occurrence and spatial-temporal clusters of diarrhea (including community childhood diarrhea data and hospital data on diarrhea caused by rotavirus and *Shigella*). Second, the study determined the relationship between diarrheal disease among children under five and identified several important risk factors, such as tubewell access, depth and arsenic levels. Additionally, simple and rapid concentration methods were developed and evaluated to detect adenovirus, a common etiologic pathogen of diarrhea in water.

The study attempted to answer the following questions: What are the trends and spatial patterns of diarrheal diseases? Are tubewells protective against diarrheal diseases? Does

arsenic mitigation by well switching raise the risk of diarrheal disease among children? The results obtained from this study provide some useful information to help policy-makers implement relevant scientific measures for diarrhea reduction and arsenic mitigation. The concentration methods developed in this study are applicable to monitor pathogens in water in Bangladesh and worldwide.

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TABLE OF CONTENTS

Page

List of Tables

List of Figures

List of Abbreviations

As:	Arsenic
CCL:	Contaminant Candidate List
CI:	Confidence interval
DOC:	Dissolved Organic Carbon
EID:	Ecology of infectious disease
GIS:	Geographic information system
HUFU:	Hollow fiber ultrafilter
ICDDR, B:	International Centre for Diarrhoeal Disease Research, Bangladesh
OR:	Odds ratio
PCR:	Polymerase chain reaction
POU:	Point of use
SES:	Socioeconomic status
TOC:	Total Organic Carbon
UNICEF:	The United Nations Children's Fund
WHO:	World Health Organization

Chapter 1

INTRODUCTION

1.1 Background

Bangladesh, a densely populated country in South Asia, has been experiencing a severe water crisis since the establishment of the country. On a territory of less than 150,000km^2, there are about 800 rivers with the total river length of around 24,140km flowing through the country, which include some regional large rivers, such as the Ganges-Brahmaputra Rivers and the Meghna River. These rivers, together with plentiful rainfall, provide adequate water resources to the population. However, the intensive agriculture, unsanitary latrines and frequent floods make the water resources vulnerable to fecal contamination (Caldwell et al., 2003). As a result, pathogen-contaminated water leads to a high prevalence of diarrheal diseases in this area. In 1970, the mortality rate of children under 5 was around 140 deaths per 1000 infants, and nearly half of the deaths were associated with diarrheal diseases (Caldwell et al., 2003). Though the number of infant deaths gradually declined in the past 4 decades, diarrheal diseases are still a leading cause of death in children under 5, accounting for 20% of all infant deaths (WHO, 2007).

Diarrheal diseases are preventable and can be remarkably reduced by providing treated water and improved sanitation infrastructure (Peterson Zwane and Kremer 2007). However, it is expensive to provide treated water and sanitation facilities in developing

countries such as Bangladesh where people mainly live in poor rural areas. To reduce diarrheal disease, during the past 30 years, people in Bangladesh have shifted their drinking water sources from surface water to groundwater, which was thought to be free of pathogens. The installation of tubewells increased rapidly, especially during the 1990s, when at least 2.5 million tubewells were installed that now provide drinking water for 95% of rural residents (Caldwell et al., 2003).

As the incidence of diarrheal diseases is declining, another health problem has emerged. During the 1990s, unsafe levels of arsenic were widely found in the shallow aquifer in Bangladesh. It was estimated that 35% of tubewells have arsenic levels above the Bangladesh permissible limit (50µg/L) and 51% had arsenic levels above the current WHO guideline (10µg/L) (BGS&MM 2000). Arsenic is a toxic metalloid responsible for many adverse health effects, such as lung and skin cancers (Kapaj et al., 2006). To date, many types of As-related diseases were reported, such as skin lesions (Tondel et al., 1999), adverse pregnancy outcomes (Ahsan et al., 2000; Milton et al., 2005), chronic bronchitis (Milton et al., 2002) and reduced intellectual function of children (Wasserman et al., 2005). The widespread arsenic contamination in groundwater puts between 35 and 77 million Bangladeshis at risk to arsenic poisoning (Argos et al., 2010; Smith et al., 2000).

Multiple arsenic mitigation efforts have been carried out with an emphasis on providing alternative water sources. Arsenic mitigation options include switching water sources to safe existing wells, switching to deep wells, using pond sand filters, and using rainwater harvesting systems (Howard et al., 2006; UNICEF 2000). Among these options, shifting to tubewells with low or no arsenic is the fastest and easiest way to get safe drinking

water (UNICEF 2000; WHO 2000). Though deep wells are difficult to install and maintain, switching to deep wells is also a good option, because deep wells commonly contain a low concentration of arsenic and have less possibility of fecal contamination. In addition to these issues, there is a growing concern that arsenic mitigation interventions may increase the water-related infectious disease burden (Lokuge et al., 2004).

1.2 Objectives

Though diarrheal disease incidence has declined over the past few decades, it remains the leading cause of death in children under 5 in Bangladesh (WHO 2007). Even worse, unsafe levels of arsenic are widely found in shallow aquifer groundwater, exposing millions of Bangladeshi. Therefore, looking for safe, convenient and sustainable drinking water resources is extremely important. However, understanding the intrinsic relationship between diarrheal diseases, arsenic levels and tubewells is urgently needed. This study focuses on Matlab, a rural area in Bangladesh, where a large dataset has been already compiled to facilitate the research project. This study attempts to provide solid scientific information to policy-makers in Bangladesh working toward diarrheal disease and arsenic mitigation. The specific aims are listed as follows:

1). To demonstrate the spatial patterns and temporal trends of diarrheal diseases.

2). To analyze the impacts of tubewell access and depth on childhood diarrhea.

3). To determine the relationship between childhood diarrhea and arsenic levels in groundwater.

4). To improve methods for detection of enteric pathogens in water by developing a simple viral concentration method for recovering adenovirus 41 in source water

5). To evaluate the efficacy of hollow fiber ultrafiltration for concentrating multiple classes of microorganisms from water.

1.3 Literature review

1.3.1 Public health significance of diarrheal diseases

Diarrhea remains the second leading cause of the mortality of children under five, which kills approximately 1.5 million children and causes 2 billion people to become sick each year globally (UNICEF and WHO 2009). Diarrhea is characterized by the increase of fluidity and frequency of fecal evacuation (Black, 1984) and in this study is defined as "the passing of 3 or more watery or loose stools during 24 hours period (UNCIEF and WHO 2009). According to the symptoms of patients, diarrhea is grouped into three major types. One is called acute watery diarrhea, which causes different degrees of dehydration. Another is called bloody diarrhea, which results in dysentery and is characterized by bloody stool. The third is persistent diarrhea, which commonly lasts more than two weeks (Keusch et al., 2006).

The etiological agents of diarrheal disease include bacteria, viruses, protozoa and helminthes. The disease is primarily transmitted through the fecal-oral route, which involves the transmission of pathogens through the environment to human host. A F-diagram drawn by Wagner and Lanoix (1958) summarized the potential routes of the transmission. Briefly, pathogens shed in feces may get into fluids, foods and on fingers and fomites, some may directly infect hosts. Flies touched with feces can carry pathogens to food or surfaces or directly transmit to hosts. Any measure that blocks the transmission routes demonstrated in the F-diagram have potentials to prevent disease transmission. For this reason, WHO (2009) recommended that in order to prevent diarrheal disease one

must focus on the following: water, sanitation and hygiene; adequate nutrition; breastfeeding; micronutrients supplements; and immunization.

1.3.2 Diarrheal diseases and water supply, sanitation and hygiene

Diarrheal diseases kill approximately 2 million people annually, yet the majority of death can be prevented through environmental interventions (Pruss-Ustum and Corvalan 2007). Globally, more than 1.1 billion people do not have access to safe drinking water and 2.6 billion individuals do not have improved sanitation facilities (WHO and UNCIEF 2004). The lack of access to improved water and sanitation is clearly linked to the number of deaths attributable to diarrheal diseases (Montgomery and Elimelech 2007).

A number of studies have suggested that interventions to improve water supply, sanitation and hygiene are beneficial to the prevention and control of diarrheal diseases (Esrey et al., 1985; Fewtrell et al., 2005; Curtis et al., 2000; Caincross et al., 2010). An early review of the effectiveness of water supply and excreta disposal improvement was written by Esrey et al. (1985). Their results suggested that well-designed projects combining water supply, sanitation and hygiene education might reduce diarrhea morbidity rate by 35-50%. Through a systematic review and meta-analysis, Fewtrell et al (2007) found that water, sanitation and hygiene interventions had similar effects on the reduction of diarrhea disease in developing countries, with the relative risk estimation ranging between 0.63 to 0.75. The results from a recent study (Cairncross et al., 2010) suggested hand washing with soap, improved water quality and excreta disposal could reduce the risk of diarrhea by 48%, 17% and 36%, respectively.

It is widely believed that poor water quality is one of the major factors related to diarrheal disease. However, studies have also found that improving the quantity of water available

might be more effective at reducing transmission of diarrheal diseases than ensuring better water quality in developing countries (Cairncross 1990). In general, adequate water supply encourages and facilitates better hygiene behaviors, in particular, frequent hand washing. This assumption was supported by findings from Nicaragua, where children with poor water access had a 34% higher rate of diarrhea than children with good water accessibility (Gorter et al., 1991). Similarly, safe excreta disposal is also a primary barrier to disease transmission. Four studies documented in China showed that safe stool disposal could reduce diarrhea morbidity by 63%, 51%, 20% and 8%, respectively (Cairncross et al., 2010). A case control study in Burkina Faso reported that risk of diarrhea among children under 3 without safe stool disposal was 50% higher than children using latrine (Teaore et al., 1941). Good personal hygiene behaviors, particularly, hand washing, can block multiple transmission routes. A number of epidemiological studies have demonstrated that hand washing remarkably reduced the morbidity of diarrhea in developing countries (Wilson et al., 1991; Hoque et al., 1986; Pinfold et al., 1996; Peterson et al., 1998). For example, a study in Thailand found that the promotion of hand washing and dish washing led to a substantial reduction of hand contamination and diarrheal diseases (Pinfold et al., 1996).

In Bangladesh, many projects have been implemented to provide safe water and sanitation and to improve hygiene practices in rural areas. The incidence of diarrhea has been declining gradually. To date, in rural areas, 95% of households obtain their drinking water from tubewells and 32% of the population uses improved sanitation facilities. The results of a five-year follow up of a water, sanitation and hygiene project showed that the improvements in environmental and hygiene practice, as measured by a lower level of

contamination of women's hands significantly reduced diarrhea prevalence (Hoque et al., 1996). However, the effects of these interventions on incidence of diarrheal disease are very complex in Bangladesh. First, though people are aware of the risk of drinking surface water, they still use it for non-drinking purposes, such as bathing. Second, the effect of water interventions is often discounted by unimproved sanitation. Results from some studies showed that children living near tubewells and who use latrines for excreta disposal have a lower incidence of diarrhea morbidity than children living near tubewells without using a latrine (Eisenberg et al., 2007; Esrey et al., 1985; Aziz et al., 1990). Moreover, arsenic contamination in groundwater discouraged people from drinking tubewell water. Therefore, many factors need be considered in order to make the best choices of safe water interventions.

1.3.3 Diarrheal diseases and socioeconomic status

Though diarrheal diseases have similarly adverse effects on rich and poor, young and old, developing countries and developed countries, its prevalence and health impacts are clearly associated with socioeconomic status (SES). The concept of SES is pervasive, which refers to "the position of individuals, families, households, or other aggregates on one or more dimensions of stratification. These dimensions include income, education, prestige, wealth, or other aspects of standing that members of society deem salient" (Bollen et al., 2001). The inverse relationship between SES and diarrhea has been illustrated in many studies. For example, D'Souza and Bhuiya (1982) assessed the SES of each household in the Matlab area using the following indicators: year of education of the head of household or mother, occupation, size of dwelling, ownership of cow and health practice. They found that the low SES groups had higher mortality rates from diarrheal

diseases whenever these indicators were included in analyses. A study in Brazil found that the number of diarrhea cases among children with low SES were two times higher than that among children with high SES, though both groups lived in a similar urban environment (Seigel et al., 1996). Emch et al (2010) found that SES had a significant impact on cholera occurrence in Bangladesh, namely, households with a higher SES score had a lower rate of cholera occurrence.

SES affects diarrheal diseases in many ways. First, people with low SES have less of a change to access improved water supply, sanitation and hygiene. Second, people with low SES often have poor nutrition and education. In addition, people with low SES have a greater change of living in poor environmental conditions and less access to health care. All of these factors lead to a higher incidence of diarrheal disease among people with low SES.

1.3.4 Microbiology of diarrheal disease

Diarrheal disease is caused by a variety of infectious microorganisms, including bacteria (e.g. enterotoxigenic *E. coli*, *Shigella*, *Vibrio cholera* and *Salmonella*), viruses (e.g. rotavirus, norovirus and adenovirus), protozoa (*Cryptosporidium*, *Giardia*) and helminthes (Keusch et al., 2006; Guerrant et al., 1990; Albert et al., 1999; Dennehy 2005). Enterotoxigenic *E. coli* and rotaviruses are two of the most frequent pathogens in developing areas. In developed countries, *Norwalk-like viruses*, *Carmpaylobacter jejuni* and *Clostridium difficile* are common pathogens causing diarrheal diseases (Guerrant et al., 1990).

E. coli is a gram-negative bacillus commonly found in the intestine of warm-blood animals. Most strains of *E. coli* are harmless, but some are associated with diseases. Five

serotypes of *E. coli* have been identified that can cause diarrhea, which are enterotoxigenic *E. coli* (ETEC), enteroinvasive *E.coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAggEC). ETEC is the most prevalent strain detected in the stools of patients who have watery diarrhea in developing areas. ETEC produces two types of enterotoxins: the LT (heat-labile) toxin and/or the ST (heat-stable) toxin. The invasion of the ST toxin in the human body may lead to secretion of fluid and electrolytes and thus cause diarrhea (Natro and Kaper 1998; Qadri et al., 2005). *Shigella* is also a very important bacterial pathogen causing diarrhea in developing countries. The genus of *Shigella* is composed of four species: *S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei. S. dysenteriae* and *S. flexneri* are two species commonly found in developing areas, while *S. sonnei* is the most common in developed countries. *Shigella* can lead to bloody diarrhea, and most patients are children under 5. Improvement of water sanitation and hygiene can greatly reduce the infection rate (Stoll et al., 1982; Keusch et al., 1989). *Vibrio cholera* is an etiological bacterium of cholera, which causes watery diarrhea and is prevalent in South Asia. Two main infectious serotypes are *V. cholerae O1* and *V. cholerae O139*. *Vibrio cholerae O1* is more frequently found in patients' stool samples in rural Bangladesh, and there is seasonal variation (Sach et al., 2003). The spatial and temporal pattern of cholera is clearly different from that of noncholera watery diarrhea in rural Bangladesh (Emch and Ali 2001).

Rotavirus is a common viral pathogen responsible for childhood diarrhea. Rotavirus is a double-stranded RNA virus and has seven serotypes. Three serotypes (A, B and C) are infectious for humans and serotype A is responsible for 90% of infections in human.

Rotavirus can cause diarrhea disease in all age groups but most commonly infects infants and young children. A two-year study of rotavirus-associated diarrhea in rural Bangladesh found that rotavirus was detected in 46.5% of stool samples of patients age 7 months to 12 months (Fun et al., 1991). Improvement of water and sanitation has not had much influence on the reduction of rotavirus-associated diarrhea. Reducing the prevalence of diarrhea and death caused by rotavirus primarily depends on vaccination of infants (Dennehy 2007).

Recently, noroviruses (NoVs) are recognized as a leading etiological agent of diarrheal disease in both developing and developed areas and affect people of all ages (Chapin et al., 2005; Dey et al., 2007; Phan et al., 2004; Vilchez et al., 2009). NoVs, previously called as "Norwalk-like viruses", are single stranded RNA viruses, which belong to the Caliciviridae family (Zheng et al., 2006). NoVs are classified into 5 genotypes and genotype I and genotype II are more frequently related to human diseases (Zheng et al., 2006). NoVs are highly contagious and can cause diseases with a few viral particles (Patel et al., 2009). Diarrhea caused by NoVs can be transmitted directly through person to person contact or through NoV- contaminated surfaces, water and food (Teunis et al., 2008). Prevention and control of NoVs-associated diarrhea mainly depends on measures that prevent people from being exposed to contaminated water and foods, strict personal hygiene and the disinfection of environmental surfaces (Patel et al., 2009).

Adenovirus is another common viral pathogen that causes diarrheal disease in both developed and developing countries. Human adenoviruses are ubiquitously found in human feces and frequently detected in water contaminated by human feces and sewage. They are double-stranded DNA viruses and classified into 6 subgroups and 51 serotypes

(Jiang 2006). An investigation of childhood diarrhea in rural Bangladesh showed that adenoviruses 40 and 41 were detected in a large proportion of patients' stool samples (Jarecki-Khan et al., 1993).

Besides the viruses mentioned above, some other viruses are also emerging as important etiological agents of diarrheal diseases, such as astroviruses, picobinaviruses, sapoviruses, and Salivirus/Klassevirus. These viruses have been reported to be responsible for diarrheal diseases among children in China, Denmark, Pakistan, Tunisia, and Vietnam (Phan et al., 2004; Olesen et al., 2005; Kirkwood et al., 2005; Nguyen et al., 2007; Shan et al., 2010).

1.3.5 Relationship between microbial indicators and pathogens

Because microbial contamination in water still leads to waterborne diseases outbreaks (Mackenzie et al., 1994; Hrudey and Hrudey 2004), monitoring pathogens in water is necessary for the protection of public health. However, it is impractical to monitor pathogens routinely, although advances in new techniques such as quantitative real time PCR (polymerase chain reaction) (*e.g*. Guy et al., 2003) and microarrays (e.g. Maynard et al., 2005) will allow detection of a large number of pathogens rapidly. Microbial indicators such as *E. coli* and enterococci are used to assess the status of fecal contamination and the risk of waterborne diseases (Yates 2007).

The criteria for an ideal indicator were initially proposed by Bonde (1966). It requires that the presence of indicators can identify the presence of pathogens in water. In addition, indicators should be correlated to health risk and have similar fate and transport characteristics to pathogens. An ideal indicator should have a strong correlation with the health hazards associated with a given type of pollution (Cabelli 1983; Dufour 1984).

The relationship between indicators and pathogens has been investigated in many studies. There is no assurance that indicators can reliably signal the presence of pathogenic contamination. *C. perfringens*, total coliforms and fecal coliforms are likely useful indicators for all three biotypes of pathogens (bacteria, viruses and protozoan). Comparatively, F-specific coliphages are better indicators for viral pathogens than total coliforms and fecal coliforms. However, *E. coli* and enterococci, two frequently used indicators, have not shown any greater likelihood of correlating with pathogens than other indicators (Wu et al., 2011).

1.3.6 Methods for detection of viral pathogens in water

Methods to detect viral pathogens in water have been developed for more than 60 years. Initial method development was prompted by public health concerns and epidemiological research on poliovirus in the natural environment (Metcalf et al., 1995). In the early 1940s, the occurrence of polioviruses in sewage was investigated in some large cities, including New York City and Chicago (Melnick, 1947). Polioviruses in sewage samples were purified and injected into monkeys, and the effects of the viruses were examined after a few weeks. Since then, methods for detecting virus have experienced a transition from observation of disease in testing animals, detection of cytopathic changes in cell cultures, immunologic assays, to molecular-based methods which directly detect viral genomes or nucleic acids (Metcalf at al., 1995).

Cell culture and PCR (polymerase chain reaction)-based methods are two that are frequently used for detecting viral pathogens in water. For cell culture, viruses are allowed to infect susceptible cell lines. After a few days of culture under proper conditions, viruses propagate in cells and produce cytopathogenic effects (CPE) which

can be observed under a microscope (Dahling 1991). This method can directly measure the infectivity of viruses in water. However, this approach is unable to detect viruses which do not infect any cell lines or do not produce cytopathogenic effects. Furthermore, the presence of toxic substances in water samples may inhibit the growth of cells (Rodriguez et al., 2009). To some extent, PCR-based methods can overcome the limitations of cell culture when detecting viruses. PCR is a powerful tool to amplify viral DNA by a series of reactions with DNA templates and primers at different temperatures. The amplified DNA can be observed by electrophoresis. The genome of RNA viruses can be amplified by reverse transcription-PCR. The theory and detailed descriptions of PCR methods can be found elsewhere (Rodriguez et al., 2009; Sambrook et al., 1989; Toze et al., 1999). Recently, real-time quantitative PCR (qPCR) has been widely used to detect viruses in water samples. The key feature of qPCR is that the copy number of viral DNA can be measured during the amplification in real time. The amount of amplified DNA is quantified in two ways: nonspecific attachment to double stranded DNA using a fluorescent dye, such as SYBR green; and specific attachment to double stranded DNA using fluorescent reporter probes. In both cases, fluorescence is generated during DNA amplification. The time when the amount of fluorescence significantly exceeds the background level (Ct value) can be used to quantify the number of DNA copies. The advantages of PCR methods lie in their specificity, sensitivity and speed. However, PCR methods do not measure the infectivity of viruses. This shortcoming can largely be overcome by using integrated cell culture and PCR methods, which have been described in some studies (Jothikumar, et al., 2005; Reynolds et al., 1996; Reynolds et al., 2004). More details are provided by Girones et al (2010).

Because viral pathogens occur in low numbers in natural water, the detection of viruses requires concentrating viruses from large volumes of water samples (e.g. 10L or more). The frequently used primary concentration approaches include adsorption-elution of viruses using charge modified filters and retention of viruses using ultrafilters. The use of nitrocellulose filters to concentrate virus was initially reported by Wallis and Melnick (1967). This microporous filtration system requires the addition of salts ($MgCl_2$ and $AlCl_3$) and adjustment of water to pH 3.5 prior to filtration (Wallis et al., 1972). Since the filter carries negative charges, it can adsorb viruses when the pH of water samples is lower than the isoelectric point of viruses of interest. Recently, the use of positively charged microporus filters to concentrate viruses from natural or tap water was developed (Sobsey et al., 1980a; Sobsey et al., 1980b). The filter, such as the 1-MDS filter, can adsorb viruses in waters near neutral pH, which could avoid steps to pretreat the sample as required by negatively charged filters. However, recovery is influenced by turbidity of the water sample and filters are relatively expensive. Recently, ultrafiltration has been applied to concentrate multiple types of viruses in large volumes (10-100L) of water, most commonly by hollow fiber ultrafilters (Morales-Morales et al., 2003; Hill et al., 2007). Ultrafilters typically have pore sizes in the range of 0.01-0.10 µm. Because of their small pore sizes, ultrafilters are capable of concentrating multiple classes of microbes simultaneously. The method, based on tangential flow hollow-fiber ultrafiltration (HFUF), has been successfully used for concentrating *Cryptosporidium* oocysts (Simmons et al., 2001; Francy et al., 2004), bacteria, viruses and parasites (Morales-Morales et al., 2003; Hill et al., 2005; Rajal et al., 2007) in drinking, surface and rain water samples.

1.3.7 Spatial data analysis and diseases mapping

The occurrence and transmission of infectious diseases is closely linked to neighborhood environment since proximity to environmental risk factors is the reason behind outbreaks. The transmission of infectious disease is more likely to take place when individuals at-risk gather in space and time. Therefore, spatial patterns of diseases shed light on the identification of potential risk factors. Since 1854, when John Snow's cholera investigation demonstrated the power of disease mapping, spatial data analysis has provided a scientific basis for developing health policy (Stolley and Lasky 1995). In the late 1990s, the advent of geographic information system (GIS) technology facilitated and advanced the development of the methods and techniques to deal with spatial data. Based on the objectives of these methods, they are divided into three main groups: exploration, visualization and modeling (Rushton, 2003).

Exploratory data analysis includes the description of spatial patterns and identification of spatial clusters. The methods for this objective include nearest neighbor analysis, spatial scan statistics, spatial autocorrelation analysis, and others. Nearest-neighbor statistics determine whether points in space are clustered, randomly distributed or dispersed. The method was initially introduced by Skellam (1952) and further developed by Clark and Evans (1954). The null hypothesis for this method is that all points are randomly distributed. To test whether the null hypothesis is to be acceptede, the ratio of expected and observed mean value of the nearest neighbor distances is measured and statistically tested by Z-statistics (Getis 1964). Spatial scan statistics (or space-time scan statistics) detect the space (time) trends of the disease data. In this method, a cylindrical window with a circular geographic base and with height corresponding to time moves in space

and time. The number of overlapping cylinders of different size and shape reflects the possible clusters of the disease data (Kulldorff et al., 2005). Spatial autocorrelation analysis aims to examine the spatial dependence of the collected disease data. If the observations are clustered in space, there is positive spatial autocorrelation and if they are dispersed in space, there is negative spatial autocorrelation. The global spatial autocorrelation is commonly measured using Moran's I or Geary's G statistics. The general concept of this method is that the similarity of the values at different locations is calculated and then weighted by the proximity of different locations (Unwin 1996).

Spatial visualization of disease data facilitates better understanding the spatial-temporal distribution of disease outbreaks. It is relatively simple to import spatial data into a GIS, reclassify the data and show key information on a map. Besides simply mapping the distribution, there are more advanced methods for disease mapping, such as kernel density estimation, spatial interpolation methods and Bayesian mapping methods. Kernel density estimation is a method used to examine disease patterns and hot spots. It generates a map showing the density of the events in a continuous field by taking the value of a specific point and spreading it across a predefined area by moving a circle with a constant radius, or bandwidth (Gatrell et al., 1996). The events distributed in the circle are weighted based on their distance to the center of the circle. Specifically, events near the center have a higher weight while events far from the center have a lower weight (Spencer and Angeles 2007). Spatial interpolation methods include inverse distance weighting (IDW), splines, and kriging. Kriging is an interpolation method that is used to estimate an unknown value from a discrete set of points with known values from a continuous field (Oliver and Webster 1990). Kriging has advantages over IDW and

splines because both predicted values and errors can be computed. There are several types of kriging, including ordinary kriging and universal kriging. A major advantage of ordinary kriging is that it can minimize the variance of the estimation error (Vann et al., 2003). Bayesian methods have been increasingly applied in disease mapping. One powerful method is the Bayesian Maximum Entropy (BME) method. The BME approach is a framework built on space/time random field (S/TRF) theory to model the uncertainty and variability of environmental variables across space and time (Christakos 1990; Christakos et al., 2002). The estimation process of this approach involves three major steps: (1) using the general knowledge to obtain the prior probability density function (PDF); (2) organizing the site-specific knowledge *S* into hard data, soft data, etc; and (3) updating the prior PDF f_G by integrating the site-specific knowledge *S* to obtain the posterior PDF at the estimated point. All information will be taken into account to produce visual maps that represent the distribution of the interested variables at any unsampled sites (Serre and Christakos 1999).

Spatial independence is a prerequisite for spatial data analysis using classical statistical methods. However, in many situations, there exists spatial dependence in observed variables. Spatial regression modeling is a method that considers the influence of spatial dependence during regression analysis. There are two primary types of spatial dependence, one is the spatial error (the error terms across different spatial units are correlated), and the other is spatial lag (the dependent variable y in place i is affected by the independent variables in both place i and j). Both types of spatial dependence can be assessed using Moran' I, an index measuring spatial autocorrelation (Anselin 2003). Based on results of model diagnostics, a spatial error model and a spatial lag model will

be run, respectively. The improved model with best fit will be chosen to predict the incidence of disease. Another spatial regression model is the geographic weighted regression (GWR) model. This model is derived from a general linear regression model (GLM). Different from GLM, each variable in GWR has a geographic coordinate. GWR is a local regression model, which emphasizes the difference in relationships between independent variables and dependent variables across space (Fotheringham et al., 2002).

In summary, this literature review briefly introduces diarrheal disease and their risk factors as well as etiological pathogens. In addition, the section briefly reviews GIS methods for studying the relationship between diarrheal diseases and their risk factors and methods for detecting causative pathogens in water. This review provided general information for understanding the 5 chapters of the dissertation.

1.4 The organization of this dissertation

This dissertation primarily focuses on childhood diarrhea in 142 villages, which is the ICDDR,B Matlab study area and explores the spatial and temporal patterns of diarrheal disease and its related risk factors. After briefly describing background and general knowledge of this body of work in chapter 1, spatial-temporal patterns of diarrheal diseases in rural Bangladesh are explored in chapter 2. In this chapter, GIS and spatial statistics are used to show the distribution and clusters of diarrheal diseases. Several risk factors of diarrheal diseases are explored including tubewell depth, As concentration and SES. Chapters 3 and 4 systematically examine risk factors associated with childhood diarrheal disease, which includes tubewell density, tubewell depth and arsenic concentration in groundwater.

Additional efforts of this dissertation are dedicated to the evaluation and development of pathogen detection methods, particularly, pathogen concentration methods. Detection of pathogens in surface water and groundwater is one of major proposed objectives for the EID project. For the detection of pathogens, adenoviruses as well as rotavirus were concentrated from 2-8 liter of tubewell water using a 0.22 µm cellulose nitrate (CN) filter. However, this membrane filtration method is unable to filter viruses in surface water because the filter is easily clogged. To improve the virus concentration method, a large pore size (8.0 µm) negatively charged membrane filter was proposed to concentrate adenovirus 41 in source water and its performance was presented in chapter 5. In addition, the efficacy of hollow fiber ultrafiltration for concentrating bacteria and viruses in groundwater was tested in the EID project (Knappett et al., 2011). It is also needed to test its efficacy for concentrating multiple classes of microbes in surface water. In chapter 6, the efficacy and the influence factors of hollow fiber ultrafiltration was examined by recovering for multiple microbes (*E. coli* KO11, *E. coli* O157:H7, Bacteriophage MS2, *Bacillus atrophaeus* spores and adenovirus 41). This chapter is complementary of the work perfomed by Knappett et al (2011).

In summary, chapters 2, 3 and 4 explore the spatial and temporal patterns of diarrheal diseases and examine risk factors related to childhood diarrhea, and chapters 5 and 6 provide supporting information about the methods used to detect diarrheal pathogens. Each chapter can be considered as an independent paper but they are also connected to one another as well as summarized here.

Chapter 2

SPATIAL-TEMPORAL PATTERNS OF DIARRHEAL DISEASES IN MATLAB, BANGLADESH

Abstract

Diarrheal diseases are a major public health problem in Bangladesh. Understanding the spatial patterns and temporal trends of diarrheal diseases is important in order to develop appropriate interventions and control measures. Geographic information systems (GIS) and spatial statistics were applied to identify spatial clusters or space-time clusters of three types of diarrheal diseases (community-collected childhood diarrhea, hospital rotavirus cases and Shigellosis cases) in Matlab, Bangladesh collected from 2000 to 2006. The results show that all three types of diarrheal diseases are clustered in space using nearest neighbor analysis. By comparing *baris* in clustered areas with unclustered areas, factors, such as tubewell depth and As content in ground water, were found to be much different in the different areas. The analysis of clusters of diarrheal diseases provides some preliminary information in order to identify risk factors of diarrheal diseases for subsequent analyses in the later chapters of this dissertation.

2.1 Introduction

Diarrhea is still one of the leading causes of childhood mortality in developing countries. Each year, approximately 1.5 million children die from diarrheal diseases globally (WHO and UNICEF 2009). Diarrhea is characterized by the increase of fluidity and frequency of

fecal evacuation (Black 1982) and results from a variety of infectious microorganisms, including bacteria (e.g. enterotoxigenic *E. coli*, *Shigella*, *vibrio cholera* and *salmonella*), viruses (e.g. rotavirus and adenovirus), protozoa (*Cryptosporidium*) and helminthes (Albert et al., 1999; Dennehy, 2005; Guerrant et al., 1990; Keusch et al., 2006). Enterotoxigenic *E. coli* and rotaviruses are the most common diarrheal pathogens in developing areas. In developed countries, *Norwalk-like viruses, Carmpaylobacter jejuni* and *Clostridium difficile* are common pathogens causing diarrheal diseases (Guerrant et al. 1990).

Bangladesh, located in South Asia, is a country with a large, dense population and poor economic conditions. Though Bangladesh has a plentiful water supply, its drinking water sources are vulnerable to fecal contamination. The pathogen-contaminated drinking water inevitably leads to childhood diarrhea. The disease kills approximately 20 per 1000 children under five (WHO 2009). A broader spectrum of etiological agents of diarrhea have been identified in surveillance in Bangladesh. Enterotoxigenic *E. coli*, rotaviruses, *Shigella* and *Carmpaylobacter jejuni*, *Vibrio cholerae* are the most common pathogens that cause diarrheal diseases (Stoll et al., 1982; Albert et al., 1999). To reduce the mortality and morbidity of diarrhea, many interventions have been implemented. One striking achievement is the installation of millions of tubewells since 1970s. Other interventions, such as vaccination, oral rehydration solution, as well as improvement of water sanitation and hygiene, have also played an important role in prevention and control of diarrhea (Hoque et al., 1996). However, the effects of these interventions are very complex and childhood mortality caused by diarrhea is still unacceptably high. To determine what factors reduce the effects of these interventions and why diarrhea is still

common, it is important to understand the spatial patterns and temporal trends of diarrheal diseases because there is a close relationship between the occurrence of diseases and neighborhood environment (Stolley and Lasky 1995). To date, spatial patterns or spatiotemporal clusters of cholera in Bangladesh have been extensively studied (Emch and Ali 2001, Emch et al., 2008, Carrel et al., 2009; Ruiz-Moreno et al., 2010). However, it is rare to have the spatial and temporal information for multiple types of diarrhea such as community-collected childhood diarrhea and hospital-collected diarrhea caused by rotavirus and *Shigella* species.

The objective of this study is to explore the spatial and spatiotemporal patterns of childhood diarrhea, and diarrhea caused by rotavirus and *Shigella* in Matlab, Bangladesh. The findings are helpful for determining risk factors of these diarrheal diseases for the subsequent analyses presented in the later chapters of this dissertation.

2.2 Methods

2.2.1 Study area

The data for this study were collected in Matlab *Thana*, Bangladesh, which is located approximately 50 km southeast of Dhaka (Figure 2.1). The study area is the field site of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) and encompasses 142 villages with a total population of 222,000 relying on 12,000 tubewells as their primary source of drinking water (Jakariya et al., 2007). An extensive Health and Demographic Surveillance System (HDSS) covering the entire study area includes monthly visits to each household to collect population and health data. Like other rural areas in Bangladesh, the main economic activities in Matlab are agriculture and fishing.

2.2.2 Data collection and management

Diarrheal disease data. Between 2000 and 2006, community health workers (CHW) visited each household every month and asked parents whether any children under five had diarrhea during the past 24 hours. The definition of diarrhea follows the method described by Black et al (1984), which is 3 or more bloody or watery stools during the previous 24 our period. Diarrhea cases were aggregated to the patrilinearly-related extended households in the study area, called *baris*, which are the unit of analysis for this study. The location and total population of each of the 10,945 *baris* in Matlab is known from HDSS records. Based on the birth date of each person, the number of children under five in each *bari* was calculated. The population within 100 meters around each *bari* was calculated as a local measure of population density.

SES and flood control. The socioeconomic status (SES) of each household was calculated using asset information collected during censuses conducted by ICDDR, B in 1996 and 2005. A categorical SES indicator was created for each household from more than 20 variables. *Bari*-level SES was calculated as the mean SES for all of the households in a *bari*. *Baris* were sorted from lowest to highest SES and then divided into five groups (Emch et al., 2010). A higher score indicates a better SES. A long embankment built in 1990 bisects Matlab into a protected area to the west side and an unprotected area to the east (Figure 2.1). About one third of the *baris* in Matlab are located within the flood control area whereas the others are unprotected.

Tubewell As. The location and depth of 10,089 tubewells in Matlab was recorded when water samples were collected for As testing in 2002-2003. Concentrations of As in tubewell water were measured using hydride generation atomic absorption spectrometry

(HG-AAS) as well as field-kits (Jakariya et al., 2007). Previous studies have shown that As concentrations in groundwater generally do not vary over time in Bangladesh, although there are exceptions especially at shallow depths (Cheng et al., 2005). In response to well testing, a considerable fraction of the population likely switched away from high-As tubewells to nearby existing low-As wells after test results were communicated. Several hundred new deep tubewells were also installed throughout Matlab as community sources of low-As water in 2005. In spite of these uncertainties, people living in *baris* are assumed to drink from the same well throughout the 2000-06 study period.

2.2.3 Spatial statistics

Nearest neighbor analysis. The nearest neighbor analysis is used to examine whether *baris* with diarrhea are clustered in space in the study area. The method was originally developed by Clarke and Evans (1954) to measure spatial relationships in plant populations and widely used in other fields because it determines whether observed points are dispersed, randomly or clustered distributed in space in a straightforward way. Using this method, an index called the nearest neighbor index (NNI) is created by measuring the ratio of the average nearest neighbor distance to the expected nearest neighbor distance based on the null hypothesis that all points are randomly distributed. Then the hypothesis is tested with Z statistic. The Z score and p value are measures of statistical significance which are used to reject null hypothesis or not. A positive value of Z score indicates the points are dispersed or randomly distributed, while a negative value indicates the points are clustered (Getis 1964). The following equations are used to calculate Z scores:

$$z = \frac{d_a - d_e}{\sqrt{\text{var}(d_i)}}, \quad \text{and } d_a = \sum_i^N d_i / N$$

Where, d_a is the average nearest neighbor distance, d_e is the expected value of the nearest neighbor distance in a random pattern, N is the number of points, d_i is the nearest neighbor distance for point i, and $\text{var}(d_i)$ is the variation of d_i. In this study, each point represents each *bari* having one or more diarrhea cases. The nearest neighbor analysis was carried out using ArcGIS 9 with ArcMap 9.2 version (ESRI, Redlands, CA).

Space-time cluster analysis. To detect clusters of diarrhea in specific local areas and specific time-periods, scan statistics were implemented to analyze space-time clusters of the point data, namely, diarrhea cases in each *bari*. Scan statistics can detect clusters either in pure time, pure space, or in space and time simultaneously. The approach creates a window (a circle or an ellipse) scanning point data across space and time, then calculates the observed value and expected value in and outside of the window (Kulldorf et al, 1997). The null hypothesis is that the observed value in the window should be equal to that outside of the window. A likelihood ratio test is used to test whether the cluster is a real cluster or due to chance. The window with maximum likelihood is the most likely cluster. The p value is obtained through Monte Carlo simulation, which assumes the observed value is a random value and the maximum likelihood is calculated. After simulating n times (for example, 999), the maximum likelihood of the observed values with that of simulated values is ranked from high to low values. The p value is the rank of the maximum likelihood of the observed value divided by the total number of the maximum likelihood values. For example, if the rank is 50, the simulation number is 999, and the total number of the maximum likelihood values is 1000 then, p=50/1000=0.05.

SaTScan software was used to detect space and space-time clusters of childhood diarrhea, and diarrhea caused by rotavirus and *Shigella*. For each type of diarrheal disease, 3 files were created separately: 1.The case file, which contains the case number of diarrhea in each *bari*; 2. The population file (for childhood diarrhea, the number of children under five in each *bari*), and 3. The location file, which contains the geographic coordinate of each *bari.* Since the diarrhea data were count data, they were assumed to have a Poisson distribution. Either purely spatial analysis or space and time analysis was carried out.

When diarrhea clusters were identified, explanatory analysis for factors related to the clusters was conducted by comparing *baris* inside to outside of the clustered area. The factors included diarrhea cases, population, population density, number of children, characteristics of tubewells, flood control and SES. Here, only childhood diarrhea clusters were analyzed because factors related to childhood diarrhea were particularly interesting. The clusters of rotavirus and *Shigella* were also analyzed using the same methods.

2.3 Results

2.3.1 Description of diarrheal diseases

Three types of diarrhea data collected during a 7 year period are presented in Table 2.1. The number of childhood diarrhea cases ranges from 4610 to 12891 with the average number of 9116. The number of cases was the highest in 2003 and gradually decreased from 2003 to 2006. The average number of rotavirus cases was 299, and was highest in 2001 with 385 cases and lowest in 2006 with 192 cases. The number of *Shigella* cases is much smaller than that of rotavirus cases, with an average of 141 cases per year.

The spatial distribution of childhood diarrhea, and diarrhea caused by rotavirus and *Shigella* during the 7 years are shown in Figure 2.2, Figure 2.3 and Figure 2.4, respectively. Childhood diarrhea cases occur throughout Matlab and diarrhea cases caused by rotavirus are mainly distributed in the central and southern parts of Matlab with very few cases in the northern area of Matlab. *Shigella* cases are centered in the southeastern area of Matlab and sporadically distributed in central and southern areas.

Monthly variations of childhood diarrhea, rotavirus and *Shigella* cases are shown in Figure 2.5 and Figure 2.6. The number of childhood diarrhea cases is higher in April in some years but there is no clear monthly trend. Rotavirus is more frequent in January and February and there is no apparent trend in diarrhea caused by *Shigella*.

2.3.2 Spatial patterns of childhood diarrheal diseases

The nearest neighbor analysis reveals that *baris* with childhood diarrheal disease were clustered in space in each year ($p<0.0001$). The NN indices calculated were all below 1 and ranged from 0.57 and 0.66. The Z scores ranged from -49.41 to -30.61 (Table 2.2). The local clusters were determined using spatial scan statistics. As shown in Figure 2.7, there is a primary cluster in southern Matlab and 8 secondary clusters in the central and northern Matlab. The location and the radius of clusters are presented in Table 2.3.

2.3.3 Space-time patterns of hospital diarrheal diseases

The spatial patterns of rotavirus and *Shigella* were also examined using nearest neighbor analysis. Rotavirus cases were spatially clustered in Matlab in each year ($p<0.001$), the NN indices ranged from 0.69 to 0.81 and the Z score ranged from -10.03 to -5.03 (Table 2.2). *Shigella* cases were spatially clustered in 2003, 2004 and 2005 but not in 2006. However, the cases might have a dispersed distribution in 2006. In 2000, 2001 and 2002,

the cases might cluster in space, but there was only about a 10% likelihood that the cases were randomly distributed.

Space-time scan statistics identified 3 clusters for rotavirus and only one for *Shigella* (Figure 2.8 and 2.9). For both types of diarrhea, the primary cluster was located in the same area and had a similar radius (Table 2.3).

2.3.4 Analysis of childhood diarrhea clusters

When diarrhea clusters were identified using space scan statistics, *baris* in the clustered area were compared with *baris* outside of the clustered area. As shown in Table 2.4, there were 2493 *baris* in the clustered areas and 4746 *baris* in the unclustered areas. The number of cases in each *bari* was much higher in that clustered area than the unclustered area. The average population and the number of children under five were similar in both areas. In *baris* protected by a flood control embankment, the number of *baris* in the clustered areas was much less than in the unclustered area. The proportion of *baris* with different levels of SES was similar in both areas. However, the proportion of tubewells with a depth below 140 feet or above 300 feet was higher in the unclustered areas than that in the clustered areas as well as the proportion of tubewells with As above 50μg/L.

2.4 Discussion

This paper analyzes the spatial patterns and temporal trends of diarrheal diseases in Matlab, Bangladesh from 2000 to 2006. Matlab was selected as the study area because health and demographic information by ICDDR, B. With these unique datasets, we are able to link diarrheal disease data to social and environmental factors through a geographic information system (GIS) and identify major factors that contribute to diarrheal disease. Childhood diarrhea was chosen in this study because children are

especially vulnerable to diarrhea, especially for children at 6-11 months, and diarrhea incidence steadily drops thereafter (Keusch et al., 2006). In addition, rotavirus and *Shigella* were also explored because these two pathogens commonly infect people in developing countries. The results of the study in Matlab can be extrapolated to other areas in the developing world that have similar environmental and social situations.

Childhood diarrhea was the highest in 2003 and then gradually dropped. This decreasing trend suggests that the health conditions for children improved with time. No decreasing trend was observed for rotavirus and *Shigella*. The reason is that the numbers of these diarrheal diseases were not very high and it is difficult to prevent exposure to these pathogens given that the water in Bangladesh was vulnerable to fecal contamination. When monthly cases of diarrhea were analyzed, no monthly trend was found for childhood diarrhea and *Shigella*. Rotavirus was more often reported in January and February, which is consistent with previous research (Bingnan et al., 1991). It is still unknown why the dry winter season is associated with rotavirus.

When a disease occurs in a population, it is important to know whether the outbreak occurs randomly or in a particular location, because this information is helpful to allocate resources and use appropriate strategies to prevent and control the diseases. Using nearest neighbor analysis, all three types of diarrheal disease were shown to cluster in space. Childhood diarrhea showed a stronger clustering pattern, followed by rotavirus and *Shigella*. These patterns are reflected by the number of diarrhea cases. Childhood diarrhea was more frequent, rotavirus was less frequent and *Shigella* was even less common.

By calculating spatial scan statistics and space-time scan statistics, 9 clusters were identified for childhood diarrhea, 3 clusters were identified for rotavirus and only 1 cluster was identified for *Shigella*. These clusters represent the hot spots of diarrhea in Matlab. The primary clusters of the three types of diarrhea were located almost in the same area with similar radii, suggesting that the three types of diarrheal diseases might have common risk factors, related to social and environmental settings where people live. To understand what factors are most important, childhood diarrhea clusters were further analyzed. Several factors, including average number of diarrhea cases, population, number of children, SES, tubewell depth and As concentration in groundwater, were compared between the clustered and unclustered areas. In the clustered area, the average diarrhea cases per *bari* was higher, while the average population and number of children were not similar. However, the tubewell depth and groundwater As concentration in the clustered area was different from those in the unclustered area. Though the significance of the difference was not statistically tested, the primary information obtained by the simple comparison could be used to judge which factors might be associated with the disease. Since more than 95% of the people in Matlab use tubewells as their drinking water source, it is not surprising that factors related to tubewells are different between the clustered and unclustered areas. Further study will be conducted to investigate the association between childhood diarrhea and tubewell depth and As concentration.

Table 2.1 Descriptive statistics of community-collected childhood diarrhea and hospital-collected diarrhea data in Matlab 142 villages from 2000 to 2006

Year	No. cases of childhood diarrhea	No. cases of rotavirus diarrhea	No. cases of *Shigella* diarrhea
2000	10091	257	101
2001	8915	385	138
2002	11248	349	152
2003	12891	299	178
2004	9776	302	192
2005	6279	306	120
2006	4610	192	103
Average	9116	299	141

Table 2.2 The nearest neighbor analysis of different types of diarrheal diseases

Type of diarrhea	Year	NN index	Z score	Significant level	Spatial pattern
Childhood diarrhea	2000	0.66	-38.68	0.000	Clustered
	2001	0.66	-38.3	0.000	clustered
	2002	0.64	-42.06	0.000	Clustered
	2003	0.65	-43.12	00000	clustered
	2004	0.57	-49.41	0.0001	clustered
	2005	0.66	-34.57	0.000	clustered
	2006	0.67	-30.61	0.000	Clustered
	2000-06	0.57	-67.19	0.000	Clustered
Rotavirus diarrhea	2000	0.78	-6.5	0.000	Clustered
	2001	0.78	-7.8	0.000	Clustered
	2002	0.71	-10.03	0.000	Clustered
	2003	0.69	-9.81	0.00	Clustered
	2004	0.69	-9.96	0.000	Clustered
	2005	0.81	-6.07	0000	Clustered
	2006	0.80	-5.03	0.000	Clustered
	2000-06	0.58	-31.29	0.000	Clustered
Shigella diarrhea	2000	0.90	-1.77	0.076	5-10% likelihood Random
	2001	0.95	-1.09	0.276	Somewhat clustered, may be due to random chance
	2002	0.95	-1.09	0.276	
	2003	0.77	-5.63	0.000	Clustered
	2004	0.82	-4.4	0.0001	Clustered
	2005	0.79	-4.36	0.0001	clustered
	2006	1.1	1.94	0.053	Dispersed, 5-10% likelihood random
	2000-06	0.7	-15.85	0.000	clustered

Table 2.3 Spatial and space-time scan statistical analysis of childhood diarrhea, rotavirus diarrhea and *Shigella* diarrhea

Type of diarrhea	Model	No. clusters	Location of clusters		Time period	p value
			Geographic coordinate	Radius (km)		
Childhood diarrhea	Retrospective purely spatial analysis with Poisson model	1	23.3420N, 90.6480E	4.17	2000-2006	0.001
		2	23.4371N, 90.7861E	1.69		0.001
		3	23.3835 N, 90.7599 E	0.88		0.001
		4	23.3963 N, 90.7312 E	0.45		0.001
		5	23.4279 N, 90.7395 E	0.20		0.001
		6	23.4067 N, 90.7602 E	0.16		0.001
		7	23.4372 N, 90.7395 E	0.15		0.003
		8	23.4079 N, 90.7553 E	0.15		0.003
		9	23.4117 N, 90.7581E	0.12		0.005
Rotavirus diarrhea	Retrospective space-time analysis with Poisson model	1	23.3262 N, 90.7264 E	4.18	2001/1/1 - 2003/12/31	0.001
		2	23.3919 N, 90.7108 E	1.97	2001/1/1 - 2003/12/31	0.001
		3	23.3463 N, 90.6404 E	0.59	2000/1/1 - 2002/12/31	0.001
Shigella diarrhea	Retrospective space-time analysis with Poisson model	1	23.3380 N, 90.7182 E	4.94	2002/1/1 - 2004/12/31	0.001

Table 2.4 Analysis of clusters of childhood diarrhea

Parameters	Unclustered area	clustered area	Ratio
Average cases per bari	0.93	1.85	0.50
Average population per bari	28	29	0.97
Average population density (n/1km2)	2860	3325	0.86
Average children number per bari	4.1	4.3	0.95
Bari number	4746	2493	1.90
No. bari protected by flood control	1926	593	3.25
No. *bari* unprotected by flood control	2820	1899	1.48
No. *bari* with the poorest SES	249	150	1.66
No. *bari* with the poorer SES	1242	666	1.86
No. *bari* with the middle SES	2086	1048	1.99
No. *bari* with the higher SES	978	527	1.86
No. *bari* with the highest SES	190	101	1.88
No. tubewell (10<depth<140ft)	2971	1122	2.65
No. tubewell (140<=depth<300ft)	1361	971	1.40
No. tubewell(depth≥300)	69	24	2.88
No. tubewell (As≤10μg/L)	933	760	1.23
No. tubewell (10<As≤10μg/L)	373	153	2.44
No. tubewell (As>50μg/L)	3094	1203	2.57

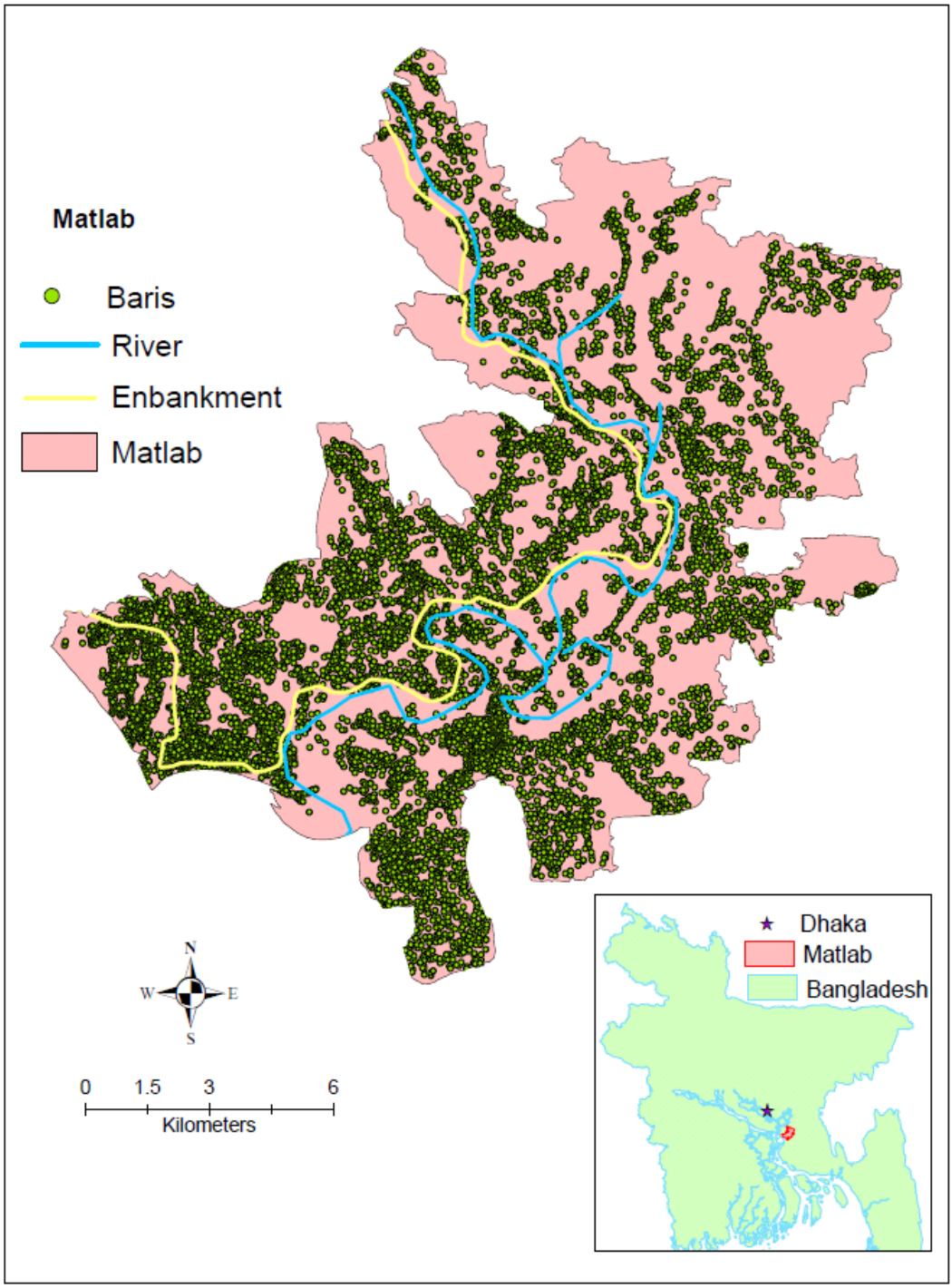


Figure 2.1 The study area: Matlab, Bangladesh

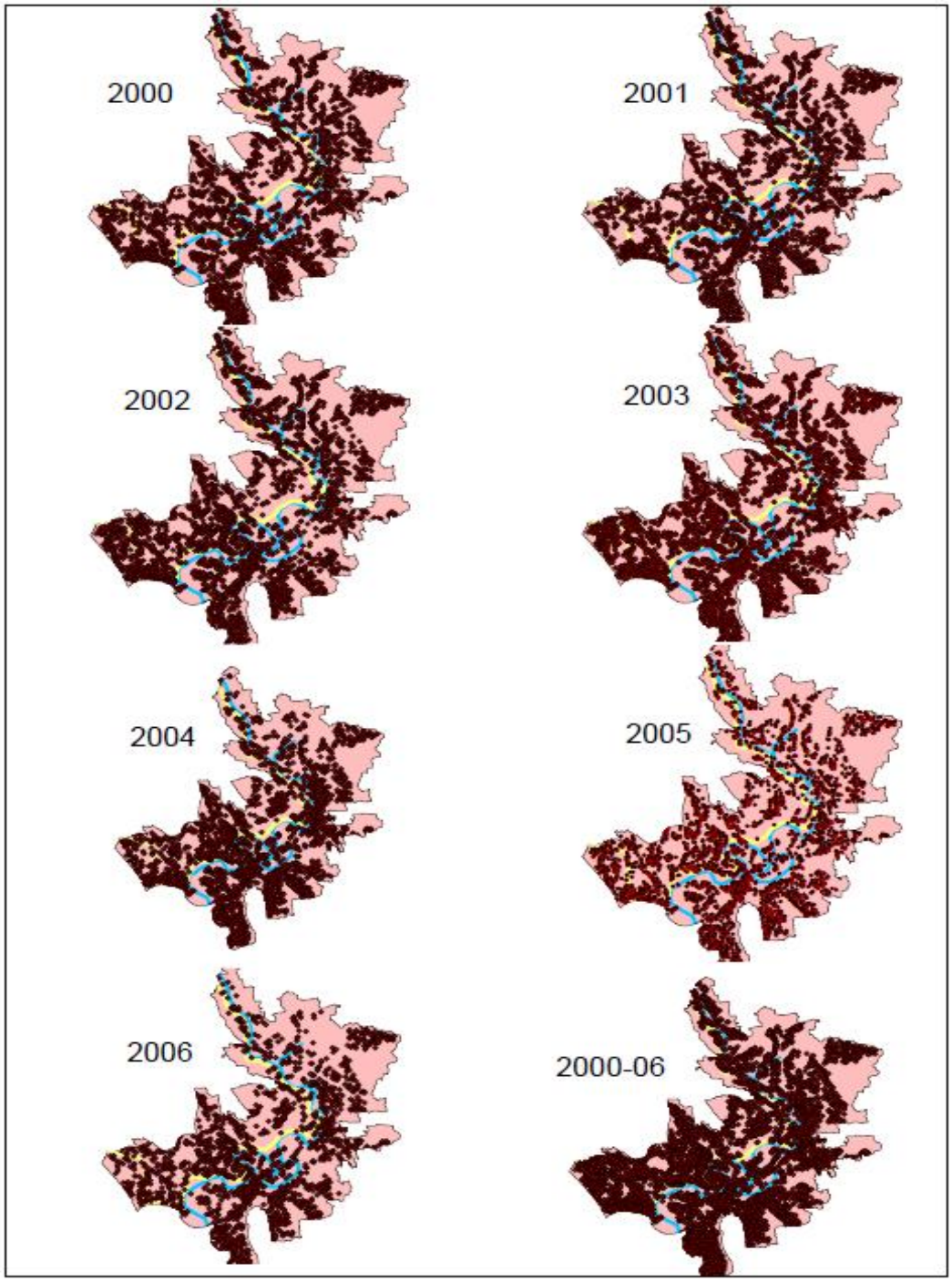


Figure 2.2 Space-time distribution of childhood diarrhea in Matlab

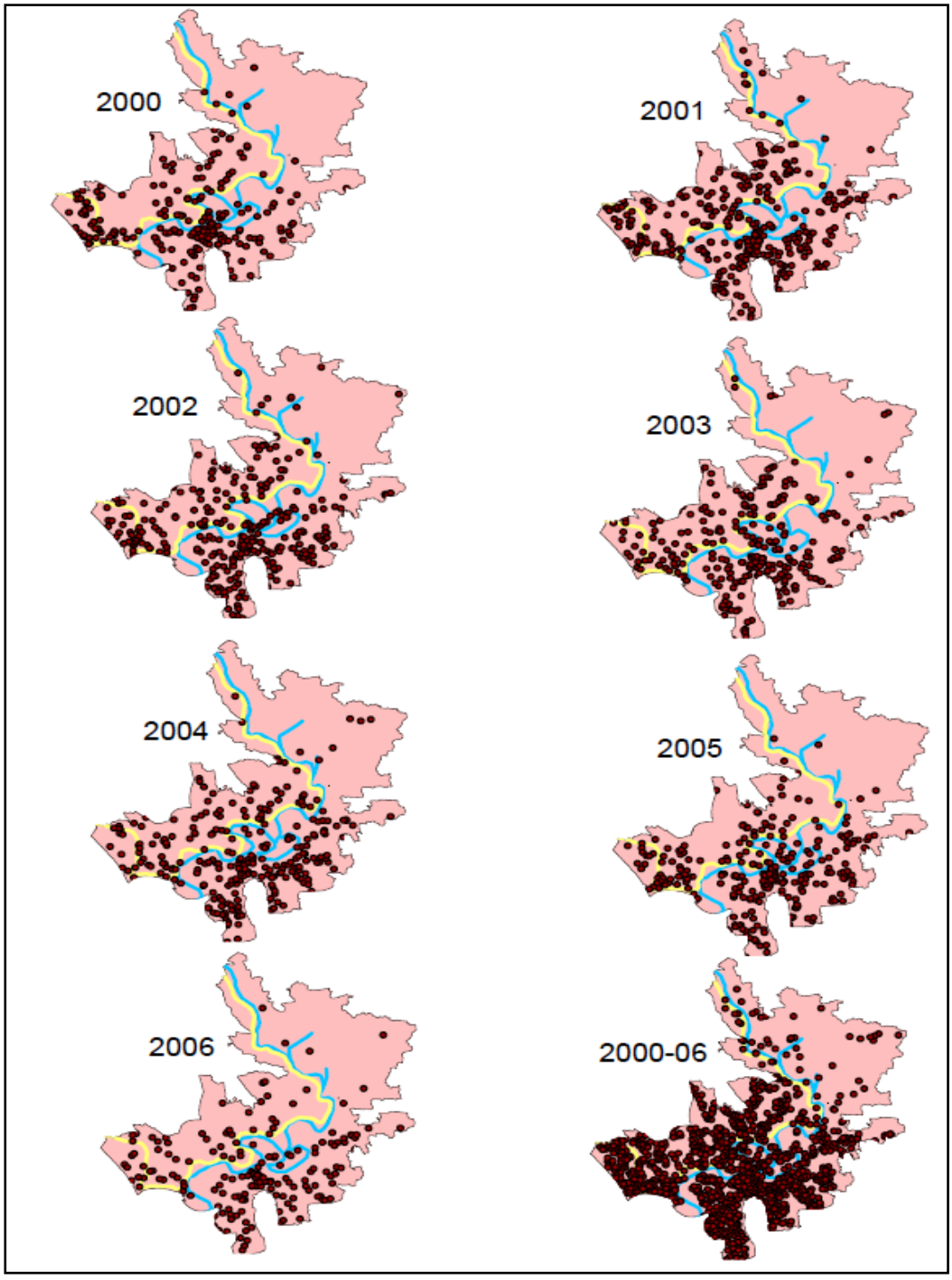


Figure 2.3 Space-time distribution of rotavirus associated diarrhea in Matlab

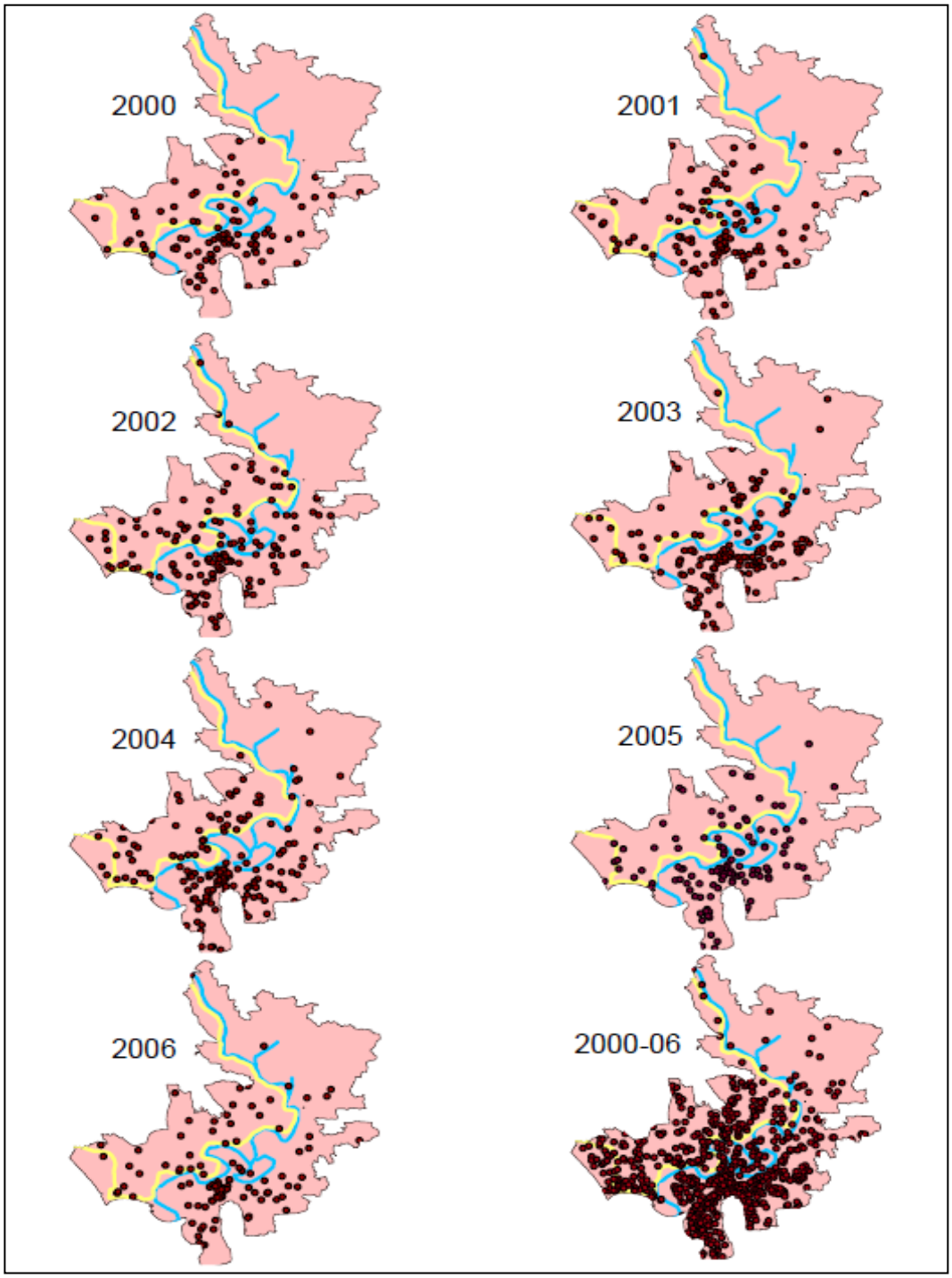


Figure 2.4 Space-time distribution of *Shigella* associated diarrhea in Matlab

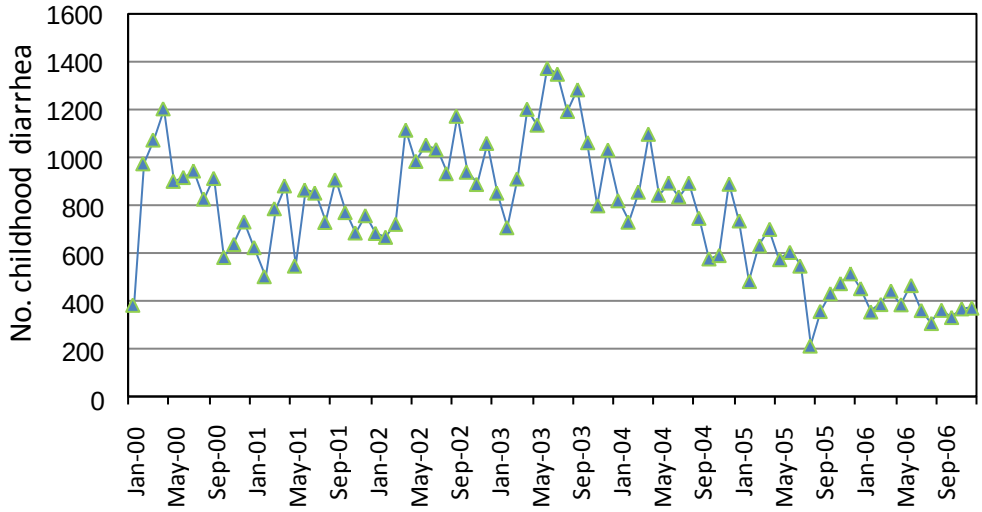


Figure 2.5 Monthly variation of the cases of childhood diarrhea in Matlab, 2000-2006

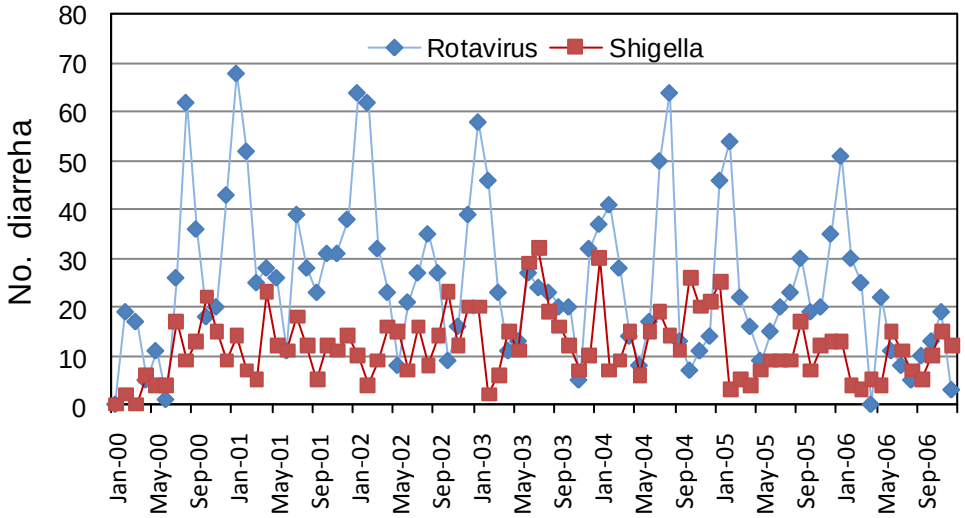


Figure 2.6 Monthly variation of the cases of rotavirus and *Shigella* diarrhea in Matlab, 2000-2006

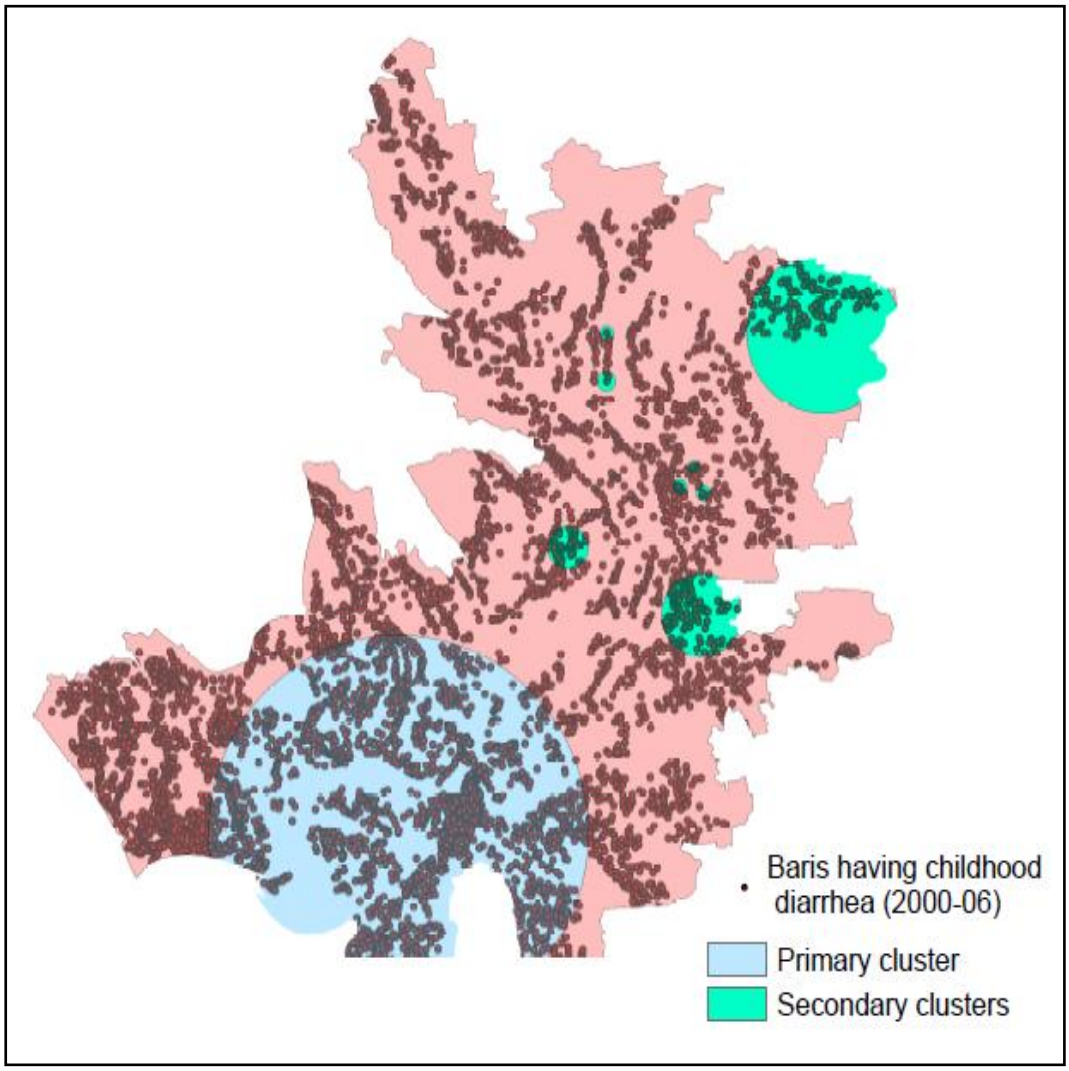


Figure 2.7 Spatial clusters of childhood diarrhea in Matab, 2000-2006

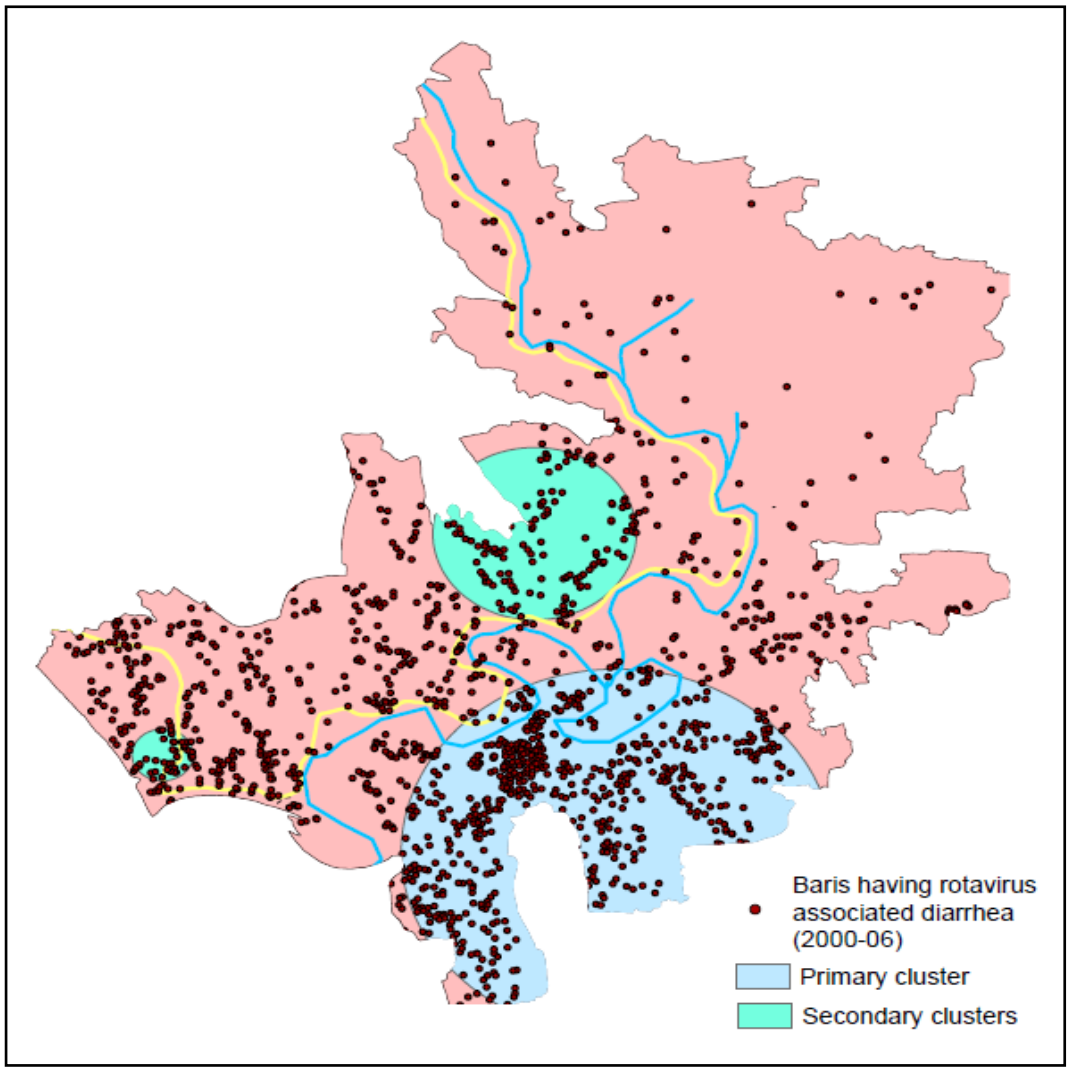


Figure 2.8 Space-Time clusters of rotavirus diarrhea in Matab, 2000-2006

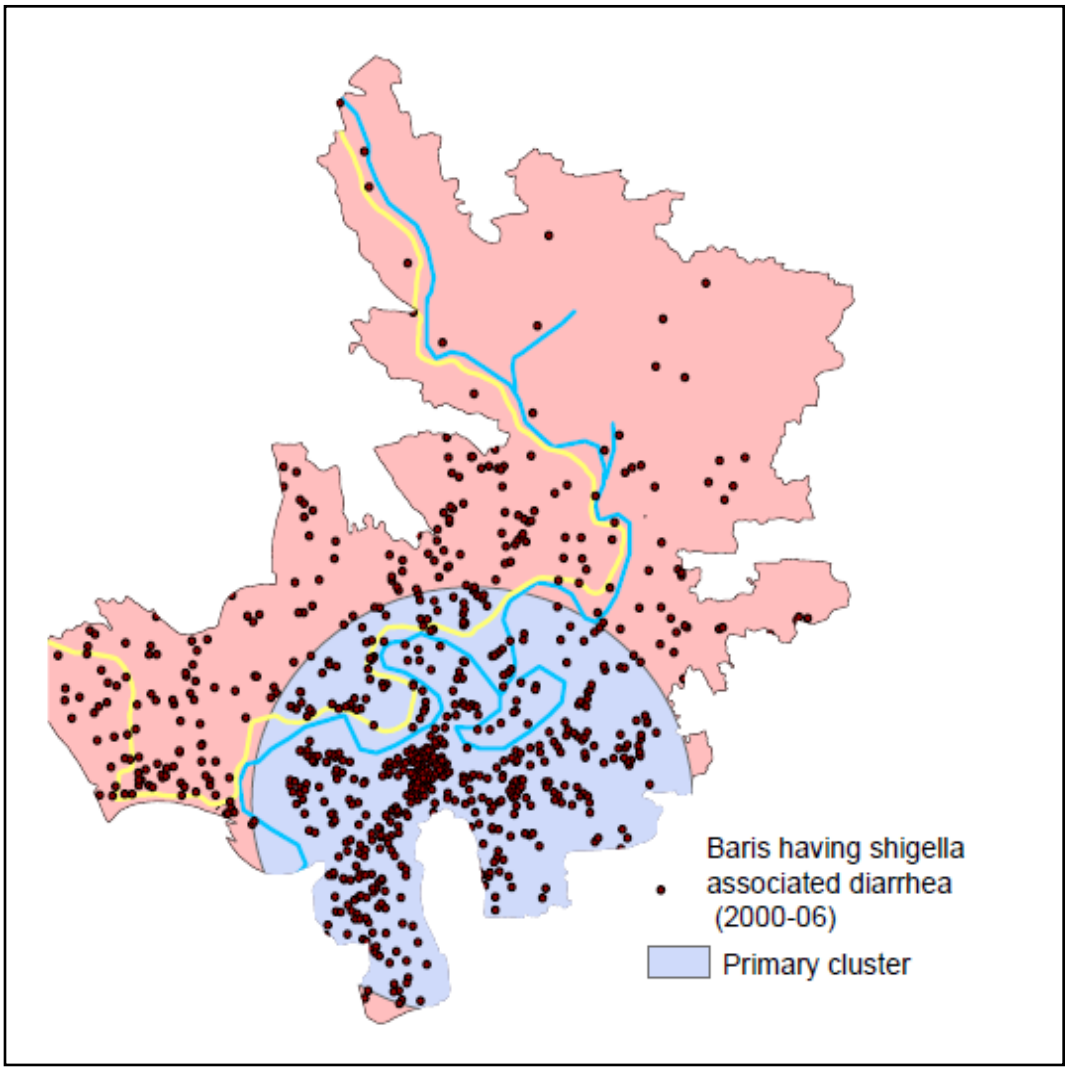


Figure 2.9 Space-Time clusters of *Shigella* diarrhea in Matab, 2000-2006

Chapter 3

IMPACT OF TUBEWELL ACCESS AND DEPTH ON CHILDHOOD DIARRHEA IN MATLAB, BANGLADESH

Abstract

During the past three decades in Bangladesh, millions of tubewells were installed to reduce diarrheal disease transmission. However, the impact of tubewells on childhood diarrhea has not been systematically examined in a large study. This study determines whether tubewell access reduces childhood diarrhea and if tubewell depth has impacts on diarrheal disease. In a community survey, information about diarrheal episodes (59,796 cases) in children under 5 was collected in 142 villages in Matlab, Bangladesh from 2000 to 2006. The location and depth of 10,089 tubewells were surveyed and integrated in a geographic information system database that was used to estimate tubewell access. Logistic regression models were built to examine the relationship between childhood diarrhea and tubewell access and depth in a bari-level (*baris* are patrilineally-related clusters of households). Socio-economic status (SES), flood-control, and population density were considered to determine if they modify the effects of access and depth. *Baris* with greater tubewell density had significantly less diarrhea with the odds ratio (OR) less than 1.00 and the 95% confidence intervals (95% CI) of 0.85-0.89. Tubewell access had a greater influence on childhood diarrhea in areas that were not protected from flooding. *Baris* using intermediate depth tubewells (140-300 feet) had more diarrheal

events (OR=1.24, 95% CI: 1.19-1.29) than those using shallow wells (10-140 feet). Households using deep wells (300-990 feet) had less diarrheal disease than those using shallow wells, however, the difference was significant only when population density was low (<1000 person/km^2). In conclusion, increased access to tubewells is associated with less childhood diarrhea but well depth, SES, and flood control also influence risk.

3.1 Introduction

Diarrheal diseases are a major public health problem in the developing world. Approximately 1.5 million children die from diarrheal diseases each year globally, which makes it the second most common cause of mortality in children under five (UNICEF and WHO 2009). Diarrheal diseases can be attributed to contaminated drinking water, poor sanitation and hygiene and more broadly to poverty (Black 1984; Peterson Zwane and Kremer 2007). In Bangladesh, diarrheal diseases are the leading cause of death in children under 5, accounting for 20% of all infant deaths (WHO 2007). In an effort to reduce diarrheal diseases, during the past 30 years Bangladesh has undertaken an almost universal shift from drinking surface water to drinking groundwater, which is generally thought to be free of microbial contamination. Millions of tubewells have been installed in Bangladesh; during the 1990s alone at least 2.5 million tubewells were installed and now provide drinking water for 95% of rural residents (Caldwell et al. 2003). Diarrheal disease incidence declined after the switch to groundwater, however, it is unknown if this decline can be attributed to tubewells because other interventions were also implemented during the same time period (Caldwell et al., 2003).

During the 1990s, high levels of arsenic were detected in shallow (typically less than 300 feet deep) aquifers in Bangladesh. According to a national survey conducted in the late

1990s (BGS/DPHE 2001), the arsenic content of groundwater in one third of the tubewells exceeded the Bangladesh permissible limit (50µg/L) and did not meet the WHO guideline of 10µg/L in half of the tubewells. Exposure to the metalloid arsenic has a number of adverse health effects including cancers of the lung, liver, skin, and bladder as well as cardiovascular disease (Smith et al., 2000; Kapaj et al., 2006; Argos et al., 2010). The widespread contamination of groundwater with arsenic puts between 35 and 77 million at risk in Bangladesh (BGS/DPHE 2001). The concentration of fecal indicator bacteria, and therefore probably waterborne pathogens, is typically orders of magnitude lower in groundwater compared to surface water in densely populated villages of Bangladesh where sanitation infrastructure is minimal (Leber et al., 2011). Nevertheless, the As crisis has forced policymakers to rethink the public health value of installing tubewells to avoid drinking microbially-contaminated surface water.

This study determines whether tubewell access reduces diarrheal disease risk and, if so, whether tubewell ≥300 ft deep are at least equally protective in terms of diarrheal diseases compared to shallow wells. This is an important issue because over 165,000 deep tubewells have been installed throughout Bangladesh to reduce exposure to As (DPHE and JICA 2010). The relationship between diarrheal diseases in children under 5 and tubewell access and depth is examined in Matlab, Bangladesh for a 7 year period.

3.2 Methods

3.2.1 Study area

The study area is Matlab, Bangladesh, which was described in Chapter 2.

3.2.2 Data collection and management

Childhood diarrhea data. This dataset was also described in Chapter 2. Briefly, CHRWs collected diarrheal disease event data for under 5 year old children for the 10,945 *baris* in Matlab from 2000 to 2006. They asked parents if their children had diarrhea within the past 24 hour recall period. Cases were also categorized as either watery or bloody. Once a case was confirmed, the illness date, birthday, and gender of the child were recorded into the HDSS database. The annual number of cases is based on parental 24 hour recall on 12 visits per year so the total disease burden would be much higher than our database shows.

Tubewell dataset. A global positioning system (GPS) survey of all 10,089 tubewells in Matlab was conducted from February 2002 to August 2004. The depth of each tubewell was determined by asking the owners; they know how deep their tubewells are because the construction price is determined by the length of PVC pipe used for the installation. Tubewell depths ranged from 10 to 995 feet. Figure 3.1 shows the depth distribution of these tubewells in 10 foot intervals. Almost two-thirds of the wells are between 10 and 140 feet deep, one third between 140 and 300 feet deep, and only 2.2% are deeper than 300 feet.

An index of tubewell access was created on the basis of the density of nearby wells. For the index based on well density, the number of tubewells within 100 m of all *baris* was first calculated. A maximum radius of 100 m was chosen because most *baris* had at least one tubewell within that distance. Three categories were then created based on the distribution tubewell density (Figure 3.1) and thus the size of each group was roughly equal: (i) <80 wells/km^2, (ii) 80 to <160/ km^2, and (iii) ≥160/ km^2. Each *bari* was given a density score ranging from 1 to 3 with higher scores representing a higher density of tubewells. Tubewells were also classified into three depth categories: (i) <140 feet, (ii)

140 to <300 feet, and (iii) ≥300 feet based on natural breaks in the distribution (Figure 3.1).

Flood control, SES and population density dataset These datasets were described in Chapter 2 and included in the analyses to determine if they modify the effect of access and depth on childhood diarrhea. A large flood control embankment was completed in 1990 which divides Matlab into a protected area with 4149 *baris* and unprotected area with 6796 *baris* (Emch, 2000; Emch and Ali, 2001). Previous studies have shown that flood control influences diarrheal disease incidence (Emch, 2000; Myaux et al., 1997). A categorical SES variable was developed using principle components analysis, creating a single household-level measure from multiple census variables (Emch et al., 2010). The SES measure reflects a composite of five variables of ownership of household assets (bed, bicycle, blanket, lamp, watch) and one ordinal variable of household wall material. Household-level SES scores were then collapsed by *bari*, and the mean score represents the *bari*-level SES. All *bari*-level SES scores were sorted from lowest to highest and divided into five categories, with a higher category reflecting a higher SES. Population as an index of *bari* size was also considered in the following statistical analysis because obviously a large *bari* has a larger population and thus has a large number of diarrhea. We used population density instead of population number in order to smooth the variability of population number in because of frequent movement of population among neighbor *baris*. Population density was calculated using total population with 100m of that *bari* divided by the area. Then it was reclassified into three groups: 1 (31≤population density<1000person/km^2), 2 (1000≤population density<3000person/km^2) and 3 (population density≥3000person/km^2). The size of each group is comparable.

A *bari*-level geographic information system (GIS) of the study area was created to link health and population data to particular *bari* locations including diarrheal disease events, population distributions, and the socio-economic status (SES) of households based on their geographic coordinates and identification numbers. Using the GIS, diarrhea cases were assigned to the nearest tubewell using a Euclidean distance measure. Especially in areas where the density of tubewells is high, the tubewell nearest to the location of a *bari* may not always be the well actually used by members of the *bari*. People likely use multiple tubewells in their neighborhood but are more likely to use the one closest to their homes.

3.2.3 Statistical analysis

Bari-level logistic regression models were built to examine the association between childhood diarrhea and well access and depth. A binary dependent variable based on the average daily diarrhea incidence was created. First, the average daily incidence was created for the entire study area using the average daily cases divided by the total number of children under 5. The average daily incidence of each *bari* (the average daily cases of a *bari* divided by the number of children in that *bari*) was then compared with the average daily incidence of the entire area. If the incidence of a *bari* was larger than the average it was assigned a value of 1 and if it was smaller then it was assigned a value of 0. Independent variables used in the models include tubewell density as a proxy for access and tubewell depth. The models for both independent variables also consider flood control, SES, and population density. When examining the relationship between tubewell depth and childhood diarreha, *baris* drinking from shallow wells (<140 feet) were the reference group which was compared to *baris* drinking from intermediate (140-300 feet)

and deep wells (≥300 feet), respectively. The association between childhood diarrhea and tubewell access and depth was indicated by an odds ratio (OR), which is the ratio of risk of a one unit increment of the independent variables. Tubewell access has 3 categories: low access=1, medium access=2 and high access=3; tubewell depth also has 3 categories but they were modeled as a dummy variable, namely, shallow wells were given a value of 0 and both intermediate and deep wells a value of 1. The 95% confidence intervals (CI) of the ORs are also reported. The logistic regression models were built using SAS 9.2 (SAS Inc., Cary, NC).

3.3 Results

3.3.1 Childhood diarrhea

Figure 3.2 shows the distribution of childhood diarrhea cases by type (bloody or watery), gender, age, and month. Approximately 90% of cases are watery and 10% bloody diarrhea in each year. Fifty-three percent of cases were in boys and 47% in girls. In each year, infants up to 1 year old had the lowest incidence. The number of cases is highest among 1 year olds and then gradually decreases through age 4. There is no clear seasonal trend to childhood diarrhea during the study period. The number of diarrhea cases during the 12 recall days each year was highest in 2003 (approximately 12,000 cases) and then gradually decreased through 2006 (approximately 4500 cases). This diarrhea curve was also illustrated by using the average daily cases and the average daily incidence (Figure 3.3). The trends of diarrhea cases and rates were consistent each year. Clearly, the average daily incidence was the highest in 2003 with 35 cases per 1000 children per day and the lowest in 2006 with only 14 cases per 1000 children per day.

3.3.2 Associations between childhood diarrhea and tubewell access

The logistic regression model showed that greater tubewell access was associated with less childhood diarrhea (OR=0.87, 95% CI: 0.85-0.89) (Table 3.1). The inverse relationship between childhood diarrhea and tubewell access was consistent in each year from 2000 to 2006, as all ORs and their 95% confidence intervals were significantly lower than 1 in the 7 years. The inverse relationship still held true when the models were controlled by both SES and population density variables. Namely, in each group of SES or population density, children in *baris* with a higher tubewell access had less likelihood to have a diarrhea. This relationship was affected by the flood control variable. Outside of the flood controlled area, the association was still significant (OR=0.84, 95% CI: 0.82-0.87). However, in the flood controlled area, the association was not significant (OR=1.02, 95% CI: 0.98-1.06).

3.3.3 Associations between childhood diarrhea and tubewell depth

The analysis of diarrheal disease and depth showed that intermediate depth wells (140-300 feet) were associated with more childhood diarrhea than shallow wells (less than 140 feet) over the entire study period (OR=1.24, 95% CI: 1.19-1.29) (Table 3.2). The association also holds for all individual year (ORs=1.15-1.35). The associations between tubewell depth and childhood diarrhea were adjusted separately by the three control variables, flood control SES and population density. Whether in a flood-controlled area or not, intermediate depth wells had a significantly higher number of cases of childhood diarrhea than shallow wells (OR>1.00, $p<0.001$). This was also true in each population density grouping. The association between diarrheal disease and well depth was only modified in the poorest (SES=1) and the richest (SES=5) *baris*, which showed that the

intermediate wells did not have a significantly higher number of cases than shallow wells (OR>1, p>0.05).

Deep wells (≥300 ft) were inversely associated with diarrhea cases compared to shallow wells in almost every year, although the relationships were not statistically significant (Table 3.3). Deep wells (≥300 ft) were not significantly associated with a change in the number of cases of diarrheal disease compared to shallow wells (OR<1.00, p>0.05) when SES was controlled, and this result was not affected whether *baris* were in the flood controlled area or outside. When population density was at the lowest level, deep wells had significantly related to lower risk of childhood diarrhea than shallow wells (OR=0.70, 95% CI: 0.51-0.96).

3.4 Discussion

Millions of tubewells have been installed throughout Bangladesh over the past several decades, mostly privately by individual households, to reduce diarrheal diseases and to access what was widely believed to be safe drinking water. Early multiple intervention trials showed that providing hand pumps and pit latrines reduced the prevalence of diarrheal diseases by half (Hoque et al., 1996). However, few studies have since attempted to examine systematically the influence of tubewell access on diarrheal diseases. To our knowledge, this is the largest and most comprehensive study of tubewell access on diarrheal diseases to date in an area where a population drinks primarily untreated groundwater. The study was made possible by the collection childhood diarrhea data for a large population (>200,000) for seven years under the community-based HDSS in Matlab. Also key for this study was the mapping of all *bari* and tubewell locations in the area with handheld GPS receivers.

One difficulty in studying the relationship between tubewells and diarrhea lies in the uncertainty of personal exposure to groundwater. To overcome this problem, an independent index of tubewell access was created, which was based on tubewell density within neighborhoods around extended households. The underlying assumption is that people living in *baris* with a higher tubewell density have better access than people in *baris* with a lower tubewell density. The very similar results derived for separate years strengthens the conclusion that tubewell access significantly reduces diarrheal disease and quantifies the extent to which it does.

There are several possible explanations for the association of greater access to tubewells with less childhood diarrhea. First, though very few inhabitants of Matlab have consumed unboiled surface water in recent years, people who have greater access to tubewells have a safer and adequate drinking water supply than alternatives such as surface water, thus, reducing diarrheal disease transmission (Esrey et al., 1985; Hoque et al., 1996) Second, people with greater access to tubewells can more easily maintain personal hygiene. This seems a more likely explanation because the bacteria counts of women who were provided hand pumps and pit latrines in early studies were lower than those in a control group (Hoque et al., 1996). Third, households with less access to tubewells might store water for longer periods of time thus increasing the chance of microbial contamination (Hoque et al., 2006).

The observation that tubewell access is more important in reducing childhood diarrhea in areas that were not flood controlled also has significant implications, especially since access is most important for poorer people in flood-prone areas. Flood-related natural disasters have been shown to be related to increased diarrheal disease risk (Ivers et al.,

2006). The data presented here suggest that the protective effect of flood control on diarrheal diseases weakens the protective effect of a nearby tubewell on diarrheal diseases. At the same time, the results suggest that installation of additional tubewells in areas without flood control where access is limited would likely further reduce diarrheal diseases.

It has been pointed out that certain forms of various arsenic mitigation interventions could result in an increase in water-related infectious diseases (Lokuge et al., 2004; Howard et al., 2006). Any optimal strategy should consider the risk of both arsenic and diarrheal diseases despite the difficulties of doing so. Response surveys have shown that approximately one-third of the population of Bangladesh exposed to high levels of arsenic has switched to a low-arsenic well (Ahmed et al., 2006). The majority of these wells in Matlab are of shallow and intermediate depth, but a growing number are deeper wells installed by the government and non-governmental organizations (NGOs) and can be routinely used by hundreds of households (van Geen et al., 2003). The present analysis did not indicate any robust relationship between the incidence of diarrheal disease for households and usage of deep wells, possibly because the number of deep tubewells is small. Their numbers have grown since the end of the study period however.

Our analysis reveals instead that drinking water from intermediate depth wells (140-300 feet) is associated with a marked increase in diarrhea relative to shallow wells (0-140 feet). In principle, the penetration of pathogens discharged by latrines and ponds into aquifers should instead decline with depth because of retention by the sediment. One possible explanation for the surprising observation reported here relates to the volume of water pumped from household wells. Households using a private well typically pump 20-

60 L over the course of a day. This corresponds to a proportion of the standing volume of water within a well that decreases from 1-3 well volumes daily for a 60-ft well to only a 0.2-0.6 well volume for a 300-ft well, assuming a 1.5" PVC pipe was used to construct a well. If pathogens can reach the standing water within a well either from above or alongside the outside of a poorly sealed well and grow within the well, more effective flushing of a shallow well compared to an intermediate-depth well could potentially reduce the level of pathogens in drinking water and therefore reduce diarrheal disease. The impact of well depth and well flushing on diarrheal diseases would not necessarily extend to wells ≥300 ft deep because those are typically community wells, rather than private wells, and are used by much larger number of household pumping 500-1000 L each day(van Geen et al., 2003).

The association between diarrhea and intermediate-depth wells is not confounded by flood control or SES even though they are both associated with childhood diarrhea. The three variables are independent of one another and there is no significant difference in flood control or SES between shallow and intermediate-depths. There is also no statistical difference in the proportion of wells in the three depth categories within and outside the embankment and the same is true for SES.

Drinking water from very deep wells could be the optimal solution for at least the next decade to both arsenic poisoning and childhood diarrhea, because deep wells are less likely contaminated by pathogens and arsenic. Since the end of the study period (December 2006), many non-governmental organizations and government programs have indeed installed, or are in the process of installing, deep wells >700 feet deep throughout the Matlab study area (and elsewhere in Bangladesh). Our finding concerning

intermediate depth wells indicates that more research is needed, however, to determine if these very deep wells are associated with a low incidence of diarrheal disease.

The groundwater arsenic problem in Bangladesh has considerably reduced access to safe drinking water in that country. Our study shows that greater access to tubewells is associated with significantly lower numbers of diarrheal diseases in children. A return to drinking untreated surface water, which contains much higher levels of microbial contaminants than even intermediate-depth wells, should therefore not be encouraged. The origin of the greater risk of diarrheal disease associated with intermediate depth tubewells needs to be investigated further. The experimental design includes the measurement of fecal indicators (e.g. *E. coli* and fecal coliform) and some pathogens (enterotoxigenic *E. coli*, *Shigella*, *Vibrio cholera*, rotaviruses, adenoviruses and noroviruses) in groundwater as well as in hand pumps. It is particularly important to determine if greater pumping is indeed required to maintain low levels of pathogens within the large volume of standing water in deeper community wells that continue to be installed to lower arsenic exposure throughout Bangladesh. Attention also should be paid to pathogens in biofilm formed in tubewell pipes.

Table 3.1 Association between childhood diarrhea and tubewell access in Matlab from 2000 to 2006.

Independent variable	Control variables	N	OR	95%CI		p
Density-based tubewell access	Unstratified	51406	0.87	0.85	0.89	<0.001
	Flood control					
	Yes	17926	1.02	0.98	1.06	0.321
	No	33480	0.84	0.82	0.87	<0.001
	Population density					
	Low	15048	0.87	0.83	0.91	<0.001
	Medium	16919	0.85	0.82	0.89	<0.001
	High	19439	0.81	0.78	0.84	<0.001
	SES					
	Low	2797	0.94	0.85	1.04	0.242
	Low medium	13357	0.84	0.81	0.88	<0.001
	Medium	21943	0.91	0.88	0.94	<0.001
	High medium	10536	0.87	0.83	0.92	<0.001
	High	2039	0.89	0.79	1.00	0.055
	Year					
	2000	7066	0.83	0.78	0.88	<0.001
	2001	7240	0.80	0.75	0.85	<0.001
	2002	7369	0.88	0.82	0.93	<0.001
	2003	7435	0.90	0.85	0.95	<0.001
	2004	7494	0.91	0.86	0.96	0.001
	2005	7445	0.93	0.87	0.99	0.014
	2006	7357	0.91	0.85	0.97	0.002

Table 3.2 The compassion of diarrhea risk between *baris* drinking intermediate tubewell water and *baris* drinking shallow tubewell water. *Baris* drinking shallow tubewell water were taken as reference

Comparison	Control variables	N	OR	95%CI		p
	Unstratified	45600	1.24	1.19	1.29	<0.001
	Flood control					
	Yes	17926	1.43	1.33	1.53	<0.001
	No	33480	1.15	1.09	1.21	<0.001
	Population density					
	Low	13157	1.25	1.16	1.36	<0.001
	Medium	15191	1.22	1.14	1.31	<0.001
	High	17252	1.25	1.17	1.33	<0.001
Intermediate	SES					
wells vs.	Low	2510	1.13	0.94	1.35	0.197
shallow wells	Low medium	11991	1.28	1.18	1.38	<0.001
	Medium	19605	1.29	1.22	1.37	<0.001
	High medium	9172	1.22	1.12	1.34	<0.001
	High	1697	1.26	0.99	1.59	0.056
	Year					
	2000	6279	1.30	1.17	1.44	<0.001
	2001	6436	1.30	1.17	1.45	<0.001
	2002	6540	1.15	1.03	1.28	0.011
	2003	6597	1.17	1.05	1.30	<0.004
	2004	6641	1.16	1.05	1.29	0.005
	2005	6590	1.35	1.21	1.50	0.001
	2006	6517	1.34	1.20	1.50	0.001

Table 3.3 The compassion of diarrhea risk between *baris* drinking deep tubewell water and *baris* drinking shallow tubewell water. *Baris* drinking shallow tubewell water were taken as reference

Comparison	Control variables	N	OR	95%CI		p
Deep wells vs. shallow wells	Unstratified	29900	0.86	0.74	1.01	0.063
	Flood control					
	Yes	11088	0.81	0.63	1.05	0.118
	No	18812	0.92	0.76	1.13	0.430
	Population density					
	Low	8599	0.70	0.51	0.96	0.028
	Medium	10407	0.94	0.72	1.23	0.670
	High	10894	0.94	0.73	1.21	0.647
	SES					
	Low	1789	1.52	0.83	2.77	0.179
	Low medium	5468	0.75	0.51	1.10	0.142
	Medium	12846	0.95	0.76	1.20	0.668
	High medium	5395	0.81	0.58	1.12	0.206
	High	1109	0.71	0.29	1.72	0.446
	Year					
	2000	4118	0.79	0.51	1.22	0.292
	2001	4227	0.85	0.55	1.31	0.462
	2002	4290	0.84	0.56	1.27	0.410
	2003	4335	0.83	0.56	1.26	0.385
	2004	4352	0.94	0.64	1.39	0.755
	2005	4316	0.79	0.51	1.22	0.286
	2006	4262	1.13	0.74	1.72	0.573

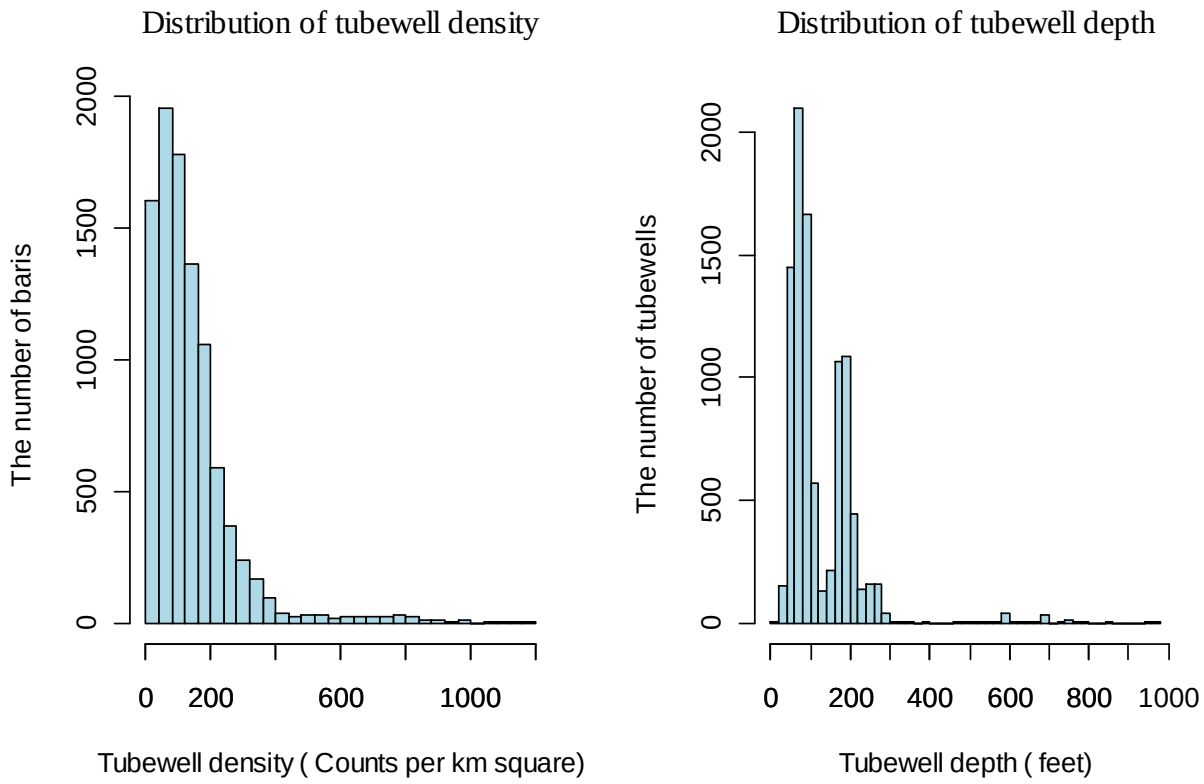
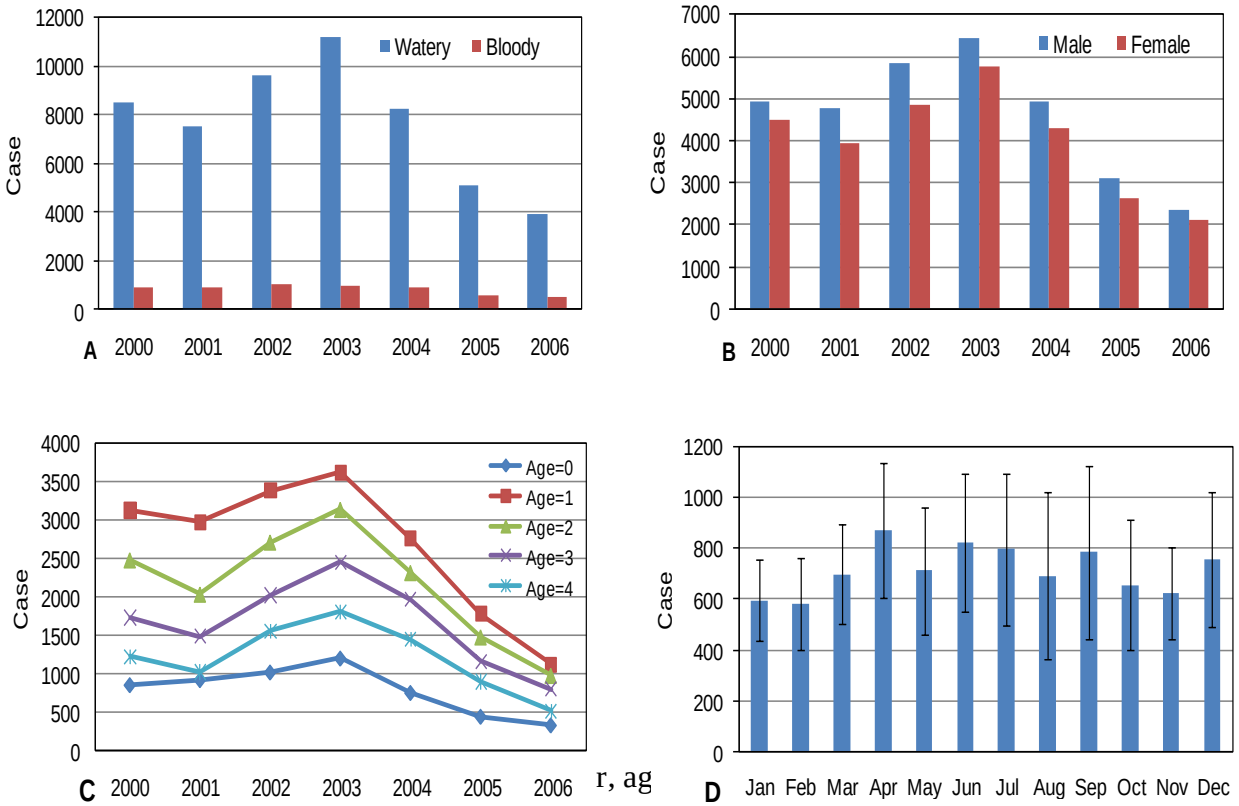


Figure 3.1 The distribution of tubewell density, tubewell depth



2000-2006. A: Watery and bloody diarrhea cases. B. Diarrhea cases by gender. C. Diarrhea cases by age. D. Diarrhea cases by month.

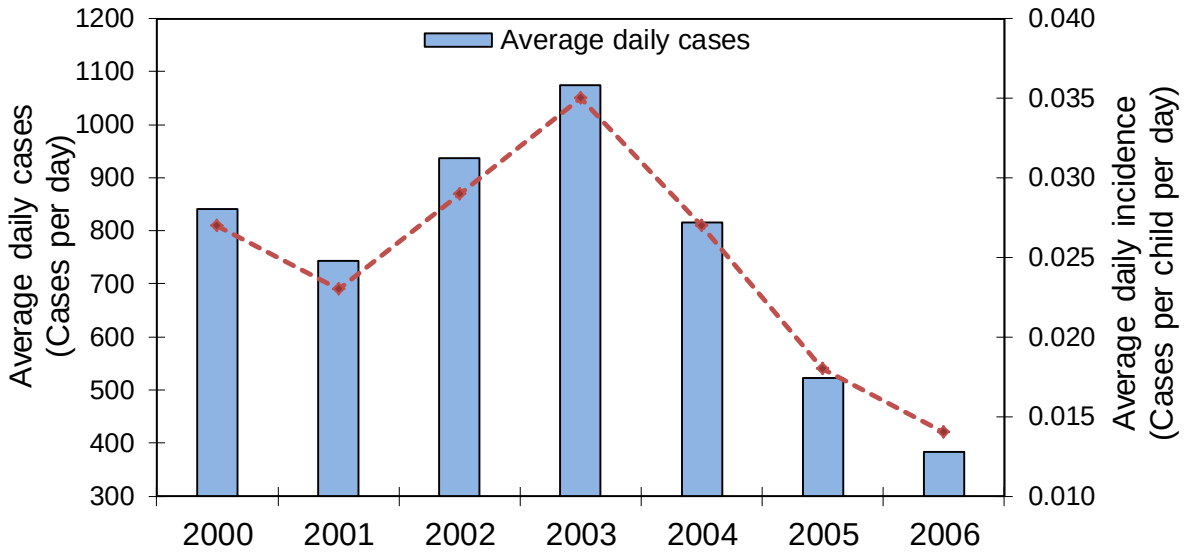


Figure 3.3 The average daily cases and incidence of childhood diarrhea in Matlab area from 2000 to 2006

Chapter 4

INCREASE IN DIARRHEAL DISEASE ASSOCIATED WITH ARSENIC MITIGATION IN BANGLADESH

Abstract

Millions of households throughout Bangladesh have been exposed to arsenic (As) levels causing various deadly diseases by drinking groundwater from shallow tubewells for the past 30 years. Well testing has been the most effective form of mitigation because it has induced massive switching from tubewells that are high (>50 µg/L) in As to neighboring wells that are low in As. A recent study has shown, however, shallow low-As wells are more likely to be contaminated with the fecal indicator *E. coli* than shallow high-As wells, suggesting that well switching might lead to an increase in diarrheal disease. Associations between childhood diarrhea and tubewell and household characteristics were evaluated using logistic regression for 60,000 episodes of diarrhea reported from 2000 through 2006. After adjusting for socio-economic status, population density, and flood control in a multivariate regression, there was an 11% ($p=0.001$) increase in the likelihood of childhood diarrhea for children drinking from shallow wells with 10-50 µg/L As compared to wells with >50 µg/L As, and a 26% increase for children under five drinking from shallow wells with $\leq$10 µg/L As. The results indicate that the health benefits of reducing As exposure are to some extent countered by an increase in childhood diarrhea risk. The findings strengthen the case for evaluating the likelihood of microbial

contamination of deeper (>300 ft) and often shared tubewells installed by the government that are typically also low in As but much less numerous than private wells.

4.1 Introduction

Diarrheal diseases and arsenic (As) poisoning are both severe health problems related to drinking water in Bangladesh (Bern et al., 1992; Kosek et al., 2003; Smith et al., 2000). High population density, frequent flooding, and poor sanitation render surface water bodies in this country particularly vulnerable to fecal contamination, thus leading to a high prevalence of diarrheal diseases, especially among children under five (Caldwell et al., 2003). In the 1970s, the infant mortality rate attributable to diarrhea was up to 36 per 1000 live births and accounted for a significant proportion of total infant mortality (140/1000) (Bern et al., 1992). Although diarrhea is still one of the leading causes of childhood mortality, the number of deaths attributed to diarrhea has decreased markedly down to 13 per 1000 infants out of total infant mortality of 65 per 1000 live births (WHO 2009). This reduction in childhood mortality reflects multiple interventions over the past several decades including advances in treatment, widely administered oral rehydration salts in particular, as well as improvements in water supply, sanitation, personal hygiene, and nutrition (de Zoysa and Feachem 1985; Esrey et al., 1985; Hoque et al., 2006; Huttly et al., 1997).

The installation of millions of tubewells that now provide drinking water to 95% of the rural population in Bangladesh most likely contributed to the decline in childhood mortality because the likelihood of microbial contamination of groundwater is much lower than that of surface water. As the number of diarrhea cases decreased, however, another health problem related to drinking water was discovered when high levels of As

were detected in many shallow tubewells in the 1990s. Until mitigation started about a decade ago, about a quarter of the population of Bangladesh was exposed to As levels above the national standard for drinking water of 50 µg/L whereas half the population was exposed above the WHO guideline for drinking water of 10 µg/L (BGS and BPHE 2001). Significant health impacts from drinking high-As groundwater have since been reported including an increase in all-cause mortality including cardio-vascular disease (Argos et al., 2010), skin lesions (Ahsan et al., 2006), various forms of cancer (Smith et al. 2000), and reduced intellectual function in exposed children (Wasserman et al., 2004). The most effective forms of As mitigation have relied on providing alternative groundwater sources to reduce exposure rather than attempts to remove As from groundwater, collect and store rainwater, or treat surface water (Ahmed et al., 2006). This is because the spatial distribution of As in groundwater is highly variable and shallow tubewells are relatively inexpensive (<$150) to install, convenient to use, and require little maintenance. Blanket testing of tubewells with field kits throughout the affected regions of Bangladesh in 2000-05 is estimated to have induced 30-50% of households with a shallow (typically <140 ft deep) high-As well to switch their consumption to a neighbor's well that is low in As, and often also shallow (Ahmed et al., 2006; Chen et al., 2007; Opar et al., 2007; van Geen et al., 2002). The next most common form of mitigation has been the installation of tens of thousands of more expensive deep (≥300 ft) community wells by the government and non-governmental organizations in villages throughout the country with a particularly high proportion of high-As wells (Ahmed et al., 2006; van Geen et al., 2003; 2007).

The possibility that some forms of As mitigation such as pond water treatment with a sand filter, rainwater harvesting, and dug wells open to surface contamination could increase the burden of diarrheal disease has been raised (Howard et al., 2006; Lokuge et al., 2004). Less attention has been paid, however, to the possibility that some categories of wells that are low in As could be particularly vulnerable to microbial contamination. The present analysis of a unique set of diarrheal disease data from Bangladesh is motivated by hydrogeological considerations suggesting that shallow tubewells that are low in As might be particularly prone to microbial contamination (Leber et al., 2010). Monthly monitoring of 125 shallow tubewells in two separate regions of Bangladesh has since confirmed that groundwater pumped from shallow low-As wells is more likely to contain the fecal indicator *E. coli* than groundwater from shallow wells that are high in As (van Geen et al., 2011). This is probably because shallow low-As aquifers are less effectively protected from shallow contaminated sources of recharge by a surface layer of fine-grained sediment often associated with shallow high-As aquifers (Aziz et al., 2008). The concern is, therefore, that the growing share of the population in Bangladesh that is switching to low-As wells, many of which are likely to be shallow, could be exposed to higher levels of fecal contamination and diarrheal disease pathogens.

4.2 Methods

4.2.1 Study area

The same as that in Chapter 2, and shown in Figure 4.1.

4.2.2 Data collection and management

Diarrheal disease data. The same as that described in Chapter 2.

SES and flood control. The same as that described in Chapter 2.

Tubewell As. The location and depth of 10,869 tubewells in Matlab was recorded when water samples were collected for As testing in 2002-2003. Concentrations of As in tubewell water were measured using hydride generation atomic absorption spectrometry (HG-AAS) as well as field-kits (Jakariya et al., 2007; Rahman et al., 2006). Previous studies have shown that As concentrations in groundwater generally do not vary over time in Bangladesh, although there are exceptions especially at shallow depth (Cheng et al., 2005). In response to well testing, a considerable fraction of the population likely switched away from high-As tubewells to nearby existing low-As wells after test results were communicated. Several hundred new deep tubewells were also installed throughout Matlab as community sources of low-As water in 2005. In spite of these uncertainties, people living in *baris* are assumed to drink from the same well throughout the 2000-06 study period.

Integration of the datasets. A geographic information system (GIS) was used to integrate the available data. A total of ~7,000 *baris* with children under five were linked to tubewells using their identification number. In brief, each *bari* has a unique identification number, and a tubewell was labeled with the identification number of a *bari* whose inhabitants drink water from this well. Among these *baris*, ~2,800 could unambiguously be assigned a single tubewell. An additional ~2,400 *baris* had two or more choices of tubewells and the remaining ~1,900 could not be assigned a tubewell using their identification number. For *baris* with more than one tubewell or without an assigned tubewell, the nearest tubewell in the database was chosen as the most likely water source for that *bari*.

4.2.3 Statistical analysis

Logistic regression models were built to examine the relationship between diarrhea diseases and arsenic as well as other variables. The dependent variable is childhood diarrhea. To create a binary dependent variable, we calculated the average daily incidence in a year using the average daily diarrhea cases (the average daily diarrhea cases=total diarrhea cases in a year divided by 12) divided by the total number of children under five. We excluded *baris* that had no children under five. We then compared the daily incidence of each *bari* with the average daily incidence. If the daily incidence of each *bari* was larger than the average, we coded the outcome as 1 and if not it was coded as 0.

Both univariate and multivariate analyses were conducted. For the univariate analysis, the As concentration of the assigned tubewell is the only independent variable and was reconstructed as a categorical variable relative to the WHO guideline for As in drinking water of 10 µg/L and the Bangladesh standard of 50 µg/L, which is also the As threshold that was used to label millions of tubewells in Bangladesh (Ahmed et al., 2006). Three ranges of tubewell As concentrations defined as very low (≤10 µg/L), low (10<As≤50 µg/L), and high (As>50 µg/L) were therefore considered in both univariate and multivariate regression models (Figure 4.1). Depth, population density, SES and flood control were first used as control variables in the univariate analyses. Well depth was classified into three categories: shallow (10<depth<140ft), intermediate (140≤ depth<300 ft) and deep (≥300 ft). The groupings reflect natural breaks in the depth distribution of wells in Matlab (Figure 3.1). Only the shallow and intermediate depth intervals are considered in this analysis because the number of deep wells is small and the vast majority (97%) have As no more than 50 µg/L (Figure 4.2). Population density was classified in three roughly equal groups: low (31-1000 per km^2); intermediate (1000-3000

per km^2) and high ($\geq$3000 per km^2). The flood control variable is binary and distinguishes *baris* that are protected by the embankment from those that are not. For the multivariate analyses, the independent variables include As level, population density, SES and flood control, whereas tubewell depth is used as a control variable, using the same categories of variables as for the univariate analysis.

Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated based on the logistic regression models and used to examine the association between diarrhea and risk factors. The "odds" is defined as the probability of an outcome event (for example, a diarrhea case) occurring divided by the probability of the event not occurring. In general, the odds ratio for a predictor of an independent variable is defined as the degree to which the odds of the outcome increase (OR>1.00) or decrease (OR<1.00) when the value of the independent variable increases by 1 unit. Here, the OR corresponds to a 1 unit change in categorical risk factorms including tubewell As (3 categories), population density (3 categories), SES (5 categories), or flood control (2 categories). A positive or negative association is indicated if the OR is significantly ($p<0.05$) larger or smaller than 1, respectively. All statistical analyses were conducted using SAS 9.2 (SAS Inc., Cary, NC).

4.3 Results

4.3.1 Descriptive statistics

The number of *baris* with children under five in Matlab remained relatively steady at ~64% of all *baris* over the 7 years spanned by this analysis (Table 4.1). The average number of children per *bari* within this group ranged from 3.7 to 4.5. The average daily incidence during the whole study period was 25 diarrhea cases per 1000 children under five. The average daily incidence varied each year with the highest rate (35 cases/ 1000 children

under five) in 2003 and the lowest rate in 2006 (14 cases/per 1000 children under five). On average, there were 3 episodes of diarrhea per *bari* per month for an average of 4 children under five per *bari* (*baris* having no children under five were excluded from the calculation).

The As content of shallow (<140 ft) tubewells is overwhelmingly high in Matlab, with only 372 (5.5%) out of 6705 wells in that depth range containing ≤50 µg/L As (Figure 2). In contrast, 3248 (83%) out of 3922 intermediate-depth wells (140-300 ft) contain ≤50 µg/L As. Shallow wells that are low or very low in As are concentrated in the central portion of Matlab, although 125 out of 142 villages in Matlab include at least one shallow well with ≤50 µg/L As (Figure 4.1). Among tubewells which were used by *baris* with children, the number of shallow wells is nearly 2 times higher than the number of intermediate-depth wells (Table 4.2).

4.3.2 Association between childhood diarrhea and As and other risk factors

Univariate logistic regression models show that the rate of childhood diarrhea decreases significantly with an increase in As levels (As was classified into 3 categories or levels, a higher As level corresponds to higher As contents) regardless of flood protection (ORs of 0.83 and 0.94, $p<0.001$) or population density (ORs ranging from 0.89 to 0.92, $p<0.001$) (Table 4.3). The rate of childhood diarrhea also decreases when As increases in all the middle category of SES. The inverse association between childhood diarrhea and tubewell As is statistically significant ($p<0.05$) for individual years, with the exception of 2004. There is a marked difference when the two depth intervals are considered separately, however. The inverse relationship between childhood diarrhea and well As is preserved for shallow wells (OR 0.92, $p=0.009$), but not for intermediate –depth wells

Multivariate regression models that consider various risk factors simultaneously are consistent with the univariate results. For shallow wells, the model continues to indicate a decrease in childhood diarrhea associated with an increase in well As (OR=0.89, p=0.001), namely, children drinking water from low-As (10-50µg/L) tubewells are 1.11 times (1.11=1/OR) more likely to have diarrhea than children drinking from high-As (>50µg/L) tubewells. Children drinking from very low-As (≤ 10µg/L) tubewells are 1.26 times ($1.26=1/OR^2$) more likely to have diarrhea than children drinking from high-As (>50µg/L) tubewells (Table 4.4). The same model shows a significant increase in childhood diarrhea with increasing population density (OR=1.15, p<0.001) and, conversely, a decrease in diarrhea with increasing SES (OR=0.90, p<0.001). There also is a significant protective effect against childhood diarrhea from flood control (OR=0.72, p<0.001). With the exception of As (OR=0.99, p=0.642), the multivariate model applied to the intermediate-depth wells shows the same direction and magnitude for the effect of all other variables including population density, SES, and flood control on childhood diarrhea (Table 4.4).

4.4 Discussion

The amount of diarrheal disease data systematically collected in Matlab over a period of seven years is to our knowledge unprecedented for a population that drinks primarily untreated groundwater. The available information provides a unique perspective on potential associations between diarrheal disease and tubewell characteristics and therefore, indirectly, groundwater quality. Conclusions drawn from this analysis should be applicable to regions throughout the world where the quality of groundwater could be compromised by a combination of high population density and poor sanitation.

Diarrheal disease data from Matlab were categorized according to the depth and As content of the tubewell that a child is most likely to drink from because of independent evidence that shallow low-As wells are likely to be particularly vulnerable to microbial contamination from concentrated shallow sources such as latrines and ponds. This prediction, based on recent data for the fecal indicator *E. coli* obtained in the context of As-related studies in Bangladesh (Leber et al., 2010; van Geen et al., 2011), is here borne out at the population level by the analysis of actual diarrheal disease patterns. A shallow source of microbial contamination is consistent with the increase in diarrheal disease in proportion to population density (van Geen et al., 2011), even if factors above ground that do not involve groundwater could also drive such a relationship. The dependence of childhood diarrhea on SES builds confidence in the analysis because it is well established that SES is a fundamental cause of many of infectious diseases (D'Souza and Bhuiya 1982; Emch et al., 2010). Children living in poor families often do not have access to clean water, are more likely to suffer from malnutrition, or may not pay much attention to personal hygiene (Keusch et al., 2006). The significant reduction in diarrheal disease associated with flood control has, to our knowledge, not been demonstrated quantitatively before. Flooding can easily disperse pathogens in water. Thus, drinking water in flood unprotected area is more vulnerable to microbial contamination. The new finding is consistent with the association of elevated *E. coli* and thermotolerant coliforms in tubewell water within a flood-prone area of Matlab (Luby et al., 2008).

The multivariate model that controls for other risk factors indicates that, on average, children under five who drink untreated groundwater from a shallow well with 10-50 µg/L As are 11% more likely to suffer from diarrheal disease relative to children drinking

from a well with >50µg/L As (Table 4.4). This is the As threshold that was used to label millions of tubewells throughout the country and that a significant proportion of households responded to by switching to a nearby well (Ahmed et al., 2006). Relative to the WHO guideline, the difference is even more striking with childhood diarrhea 26% more likely for children drinking from a shallow well with $\leq$10 µg/L compared to wells with >50 µg/L. This is consistent with the highest difference in frequency of *E. coli* detection previously reported for shallow wells meeting the WHO guideline for As compared to wells with >50 µg/L (van Geen et al., 2011).

Comparing the relative risk of the resulting increase in diarrheal disease to prolonged exposure to As is beyond the scope of this study (Lokuge et al., 2006; Howard et al., 2006), but the outcome of such an analysis could be that switching to a low-As well, even if it is shallow, remains the preferred course of action. Potential contamination of groundwater from any depth during storage in the home should be taken into account (Hoque et al., 2008), although this particular confounding factor evidently did not overwhelm the relation between diarrheal disease and As, a characteristic of shallow tubewells that can now be plausibly linked to the microbial quality of groundwater.

The average daily incidence of 25 episodes of diarrheal disease per 1000 children under five recorded over twelve 24 hour periods translated into 0.75 episodes per child per month and 3 episodes per *bari* per month based on the average number of 4 children per *bari* (Table 4.1). Beyond the issue of As, our analysis therefore shows that the microbial quality of groundwater could be a significant factor contributing to the continuing high incidence of diarrheal disease in Bangladesh and other countries with high population density, poor sanitation, and similar aquifer geology.

The lack of a relationship between childhood diarrhea and As for intermediate wells suggests that, unlike shallow wells, there may not be a pathway of fecal contamination that leads from latrines and ponds to intermediate-depth wells. At the same time, a separate analysis of the same Matlab data that considers population density and well depth only has shown that intermediate-depth wells are on average associated with significantly higher levels of childhood diarrhea than either shallow wells or deep wells, regardless of their As content. The reason for this association is presently unknown. The implication is that, in Matlab at least, households may have to be discouraged from switching to a low-As well in the intermediate-depth range if either a shallow or a deep low-As well is available within walking distance. Another analysis of disease data within a smaller area of Matlab where a more detailed survey was conducted at the household level has shown that switching to a very deep well (>700 ft) is accompanied by a significant reduction in diarrhea (Escamilla et al. unpublished data). The implication is that a deep well that is low in As is the preferred option overall for reducing As exposure, followed by a shallow well that is low in As if no alternative is available, and lastly an intermediate-depth well that is low in As.

One concern is the interaction effect between As exposure and microbial contamination on childhood diarrhea. It was found that prenatal arsenic exposure might be harmful for the immune system development and cause immune suppression or deficiency (Raqib et al., 2009). Therefore, children who drink As contaminated groundwater might be susceptible to infection of pathogens in water and more likely to have diarrhea (Rahman et al., 2011). However, in this study, we found As levels in groundwater to be negatively associated with childhood diarrhea. There was an inverse relationship between As content

and the concentration of fecal indicator bacteria in groundwater which is controlled by hydrogeological characteristics. Even if there is an interaction between As and microbial contamination in groundwater, the effect is subtle compared with the influence of hydrogeology in shallow aquifer.

Our study has a few limitations. First, for a significant proportion of *baris*, assignment of a specific tubewell was based on Euclidian distance between the centroid of the area covered by the *bari* and the location of the nearest tubewell. In some cases, the closest tubewell may not be the one people charged with collecting water go to if that well is owned by a neighboring *bari*. To address this problem, we examined the 2800 *baris* for which a tubewell was unambiguously assigned using the same multivariate logistic regression analysis. The results show again a significant inverse association between childhood diarrhea and As content in shallow wells (OR=0.88, p=0.023), but not in intermediate-depth wells (OR=1.03, p=0.438) (Table 4.5). A significant proportion of households probably also switched their source of drinking water after their well was tested for As and deep low-As community wells were installed, and this could have affected diarrheal disease patterns. However, the inverse association between childhood diarrhea and As contents in groundwater hold true consistently both before (2000-2002) and after (2005-2006) the As testing campaign (Table 4.6).

In spite of these limitations, we believe that the inverse relationship between the As content of shallow wells and the likelihood of diarrheal disease is an important finding and should be considered in future planning efforts to reduce As exposure. The patterns of diarrheal disease associated with other depth intervals suggests that deep community

wells should eventually be installed even in those villages where there is a significant proportion of low-As well that are either shallow or intermediate in depth.

According to the results from chapter 2 and chapter 3, deep wells as a safe drinking water source should be a primary choice. However, the number of deep wells is very small, any shallow wells or intermediate-depth wells which are low in As and pathogens may be considered. Therefore, testing As and pathogens in tubewell water is necessary to make sure that the water from these wells is safe to drink. Recently, point-of-use (POU) water treatment has emerged as a promising approach to provide safe drinking water directly to households in developing countries and greatly reduce the incidence of diarrheal diseases (Souter et al., 2003; Sobsey et al., 2008). Some POU water treatment systems (for example, a promising system based on flocculation, sedimentation and disinfection), have been demonstrated to effectively remove bacterial, viral and parasitic pathogens as well as arsenic from drinking water (Souter et al., 2003). The promotion and scaling up of such POU systems may provide an alternative solution for people in Bangladesh to accessing to safe drinking water.

Table4.1 Population and disease summaries in 142 Matlab villages

Year	Total population	No. *baris* having children	No. children under five	Total No. diarrhea cases	Average daily diarrhea cases	Average daily incidence
2000	217772	7066	31586	10091	841	0.027
2001	219579	7240	31776	8915	743	0.023
2002	221810	7369	31808	11248	937	0.029
2003	223443	7435	31077	12891	1074	0.035
2004	223573	7494	30099	9776	815	0.027
2005	222983	7445	28537	6279	523	0.018
2006	221527	7357	26958	4610	384	0.014
Average	221527	7344	30263	9116	760	0.025

Note: Average daily diarrhea cases= total number of diarrhea cases/12 based on the diarrhea were collected by 12 visits (days) each year.
Average daily incidence= Average daily diarrhea cases/ the number of children under five.

Table 4.2 Number of shallow and intermediate depth tubewells in *baris* with one or more children

	10-140ft	140-300 ft
As≤10µg/L	116	1510
10<As≤50µg/L	123	407
As>50µg/L	3920	439

Table 4.3 Univariate analysis of the association between childhood diarrhea and tubewell arsenic

Control variables	n	p	OR	95%CI
Depth (ft)				
10-140	29111	0.009	0.92	0.86-0.98
140-300	16489	0.594	0.99	0.95-1.03
Flood control				
Yes	16863	<0.001	0.83	0.80-0.87
No	28737	<0.001	0.94	0.91-0.96
Population density				
0-1000	13157	<0.001	0.89	0.85-0.93
1000-3000	15191	<0.001	0.92	0.88-0.95
≥3000	17252	<0.001	0.90	0.87-0.93
SES quintile				
1 (poorest)	2510	0.063	0.91	0.82-1.01
2	11991	<0.001	0.91	0.87-0.95
3	19605	<0.001	0.90	0.87-0.93
4	9172	<0.001	0.86	0.82-0.91
5 (richest)	1697	0.096	0.90	0.79-1.02
Year				
2000	6279	<0.001	0.87	0.82-0.93
2001	6436	<0.001	0.89	0.84-0.95
2002	6540	0.001	0.91	0.86-0.96
2003	6597	0.011	0.93	0.88-0.98
2004	6641	0.071	0.95	0.90-1.01
2005	6590	<0.001	0.88	0.83-0.93
2006	6517	<0.001	0.85	0.80-0.91

Odds ratios reflect the likelihood of disease for children drinking from wells with low As (10-50 µg/L) relative to wells with very low As (≤10 µg/L), or for children drinking from wells with high As (>50 µg/L) relative to wells with low As (10-50 µg/L).

Table 4.4 Multivariate analysis of associations between childhood diarrhea and arsenic, population density, SES and flood control

Control variables	Independent variables	n	P	OR	95%CI
0<depth <140 ft	As	28654	0.001	0.89	0.84-0.96
	Flood control		<0.001	0.72	0.68-0.76
	Population density		<0.001	1.15	1.11-1.18
	SES		<0.001	0.90	0.87-0.92
140≤depth <300ft	As	16321	0.642	0.99	0.95-1.03
	Flood control		<0.001	0.89	0.83-0.95
	Population density		<0.001	1.15	1.10-1.19
	SES		<0.001	0.87	0.84-0.90

Table 4.5 The association between childhood diarrhea and arsenic, population density, SES and flood control for *baris* which a tubewell was unambiguously assigned in 142 villages

	Independent variables	*Baris* with matched wells in 142 villages			
		n	p	OR	95%CI
Shallow wells (10<depth <140 ft)	As	11617	0.023	0.88	0.78-0.98
	Flood control		<0.001	0.67	0.61-0.73
	Population density		0.001	1.08	1.03-1.14
	SES		<0.001	0.88	0.84-0.92
Intermediate-depth wells (140≤depth <300ft)	As	5651	0.438	1.03	0.96-1.11
	Flood control		0.506	0.96	0.86-1.08
	Population density		0.004	1.10	1.03-1.18
	SES		<0.001	0.85	0.80-0.90

Table 4.6 The association between childhood diarrhea and As in different time periods after adjusting flood control, population density and socioeconomic status.

Time period	Depth	n	p	OR	95%CI of OR
2000-2002	Shallow wells	12027	0.017	0.88	0.90-0.98
	Intermediate-depth wells	6848	0.259	0.97	0.91-1.03
2004-2006	Shallow wells	12474	0.014	0.88	0.79-097
	Intermediate-depth wells	5651	0.496	1.02	0.96-1.09

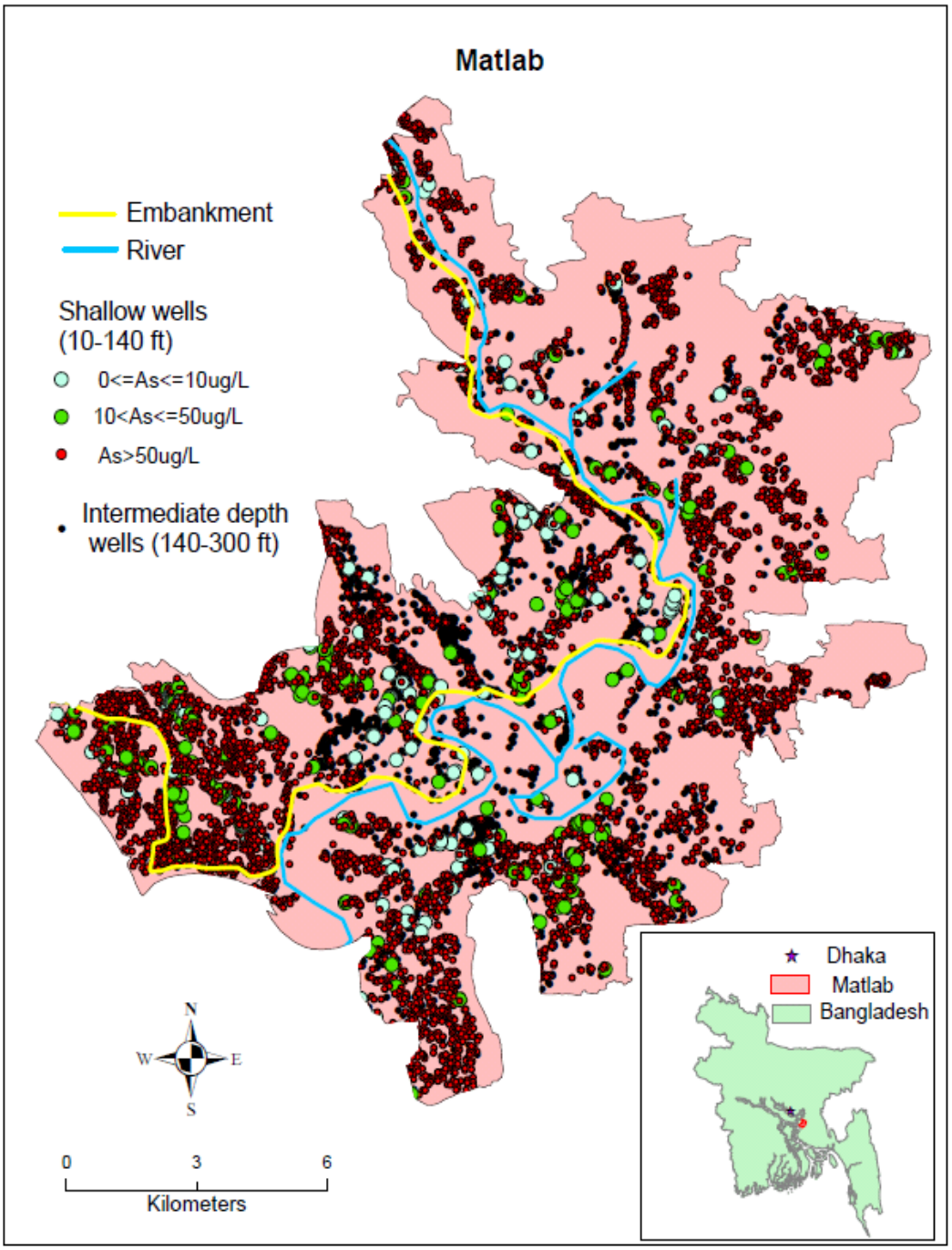


Figure 4.1 Matlab, Bangladesh study area and the spatial distribution of tubewells at each As and depth interval

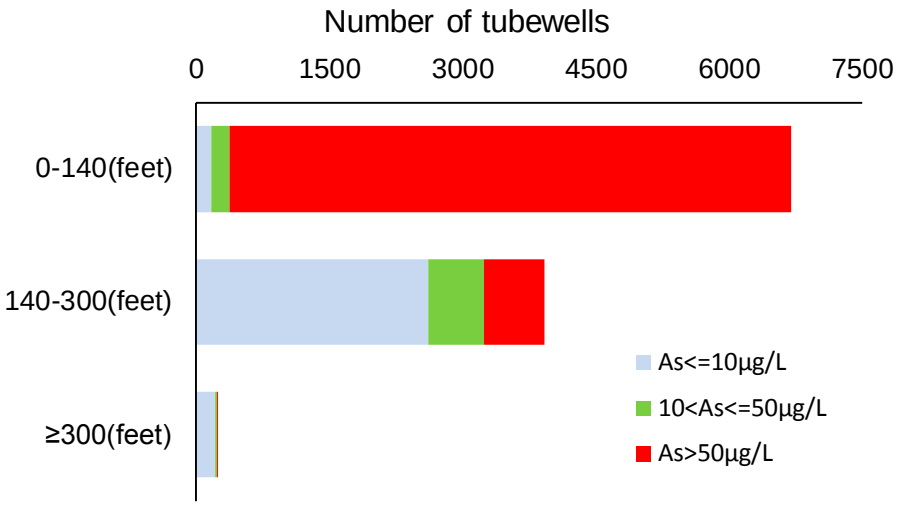


Figure 4.2 Frequency distribution tubewells at each As and depth interval.

Chapter 5

A SIMPLE AND NOVEL METHOD FOR RECOVERING ADENOVIRUS 41 IN SMALL VOLUMES OF SOURCE WATER

(Published in *Journal of Applied Microbiology*, 2011, 110:1332-1340)

Abstract

The study described the development of a new procedure to recover viruses in small volumes (1L) of water samples based on adsorption, elution and evaporation. One liter of source water seeded with adenovirus 41 was adjusted to pH 3.5 and filtered using a large pore size (8.0 µm) negatively charged membrane filter (SCWP, 47mm diameter). Then, the filter was eluted using 4 mL of 1.5% beef extract plus 0.75% glycerol (pH=9.0). The eluate was reconcentrated to 0.1 mL or less volumes through evaporation assisted with air flow and heating at 55°C. Recovery of adenoviruses reached 55% under tested conditions and reduced filtration time by 85% in contrast to the widely used small pore size filter (0.45 µm pore size, 47 mm diameter). Reconcentration by evaporation achieved approximately 86.8% recovery from source water in approximately 1 hour at no cost. In conclusion, the virus concentration method developed in this study is simple and cost-effective, and can be used to efficiently recover adenoviruses from turbid water samples.

5. 1 Introduction

Human enteric viruses are responsible for a large proportion (30%-90%) of gastroenteritis cases worldwide (Gerba et al., 1985; van Heerden et al., 2005). These

viruses include enteroviruses, noroviruses, hepatitis A virus, adenoviruses, rotaviruses and astroviruses. They are constantly found in raw sewage of large populations and in environmental waters impacted by fecal contamination, sometimes leading to waterborne outbreaks (Leclerc et al., 2002; Reynolds et al., 2008). Human adenoviruses in particular are frequently detected in water and are potentially a good indicator for human viral fecal contamination (Jiang 2006; Wong et al., 2009). Human adenoviruses are double stranded DNA viruses and consist of 51 serotypes which are divided into 6 subgroups (A to F subgroup). Among these serotypes, adenovirus 40 and 41 of subgroup F are responsible for the most cases of childhood diarrheal disease (Jiang 2006). For example, a study in Bangladesh reported that adenovirus 40 and 41 were responsible for 40.8% and 59.2% of adenoviruses caused diarrheal diseases, respectively (Jarecki-Khan et al., 1993). Because the presence of human adenoviruses in drinking water is a threat to public health, they are identified as emerging contaminants in U.S. EPA's Contaminant Candidate List (CCL).

Studies have shown that enteric viruses can cause diseases even at low doses when ingested (Bosch 1998). To protect public health, the development of sensitive methods is required to detect these viruses at low concentrations in water samples. In general, viruses are concentrated from large volumes of water samples by multiple concentration steps. For primary concentration of viruses from water, the commonly used approaches are adsorption-elution of viruses using negatively or positively charged filters or virus retention by pore size exclusion using ultrafilters. The first practical microporous filtration system was developed by Wallis and Melnick (1967) using nitrocellulose filters (negatively charged) requiring the addition of salts ($MgCl_2$ or $AlCl_3$) and adjustment of water to pH 3.5 prior to filtration to achieve virus adsorption (Wallis et al., 1972). These

negatively charged filters can adsorb viruses when the pH of the sample is lower than the isoelectric point of the viruses, so that the electrostatic potential of the viral capsid is more positive. The use of positively charged microporous filters is another adsorption-elution approach to concentrate virus from natural or tap water (Sobsey et al. 1980a, 1980b). The advantage of using positively charged filters such as the Virosorb 1-MDS filters (CUNO, Inc., Meriden, CT) is that viruses can be concentrated from waters near neutral pH, without pretreatment of the sample prior to filtration (Sobsey and Jones. 1979). The disadvantage is that virus recovery is severely affected by the salt concentration and turbidity of the water. Furthermore 1-MDS filters are relatively expensive (>$150 USD each). Recently, ultrafiltration has been applied to concentrate multiple types of viruses in large volumes (10-100 L) of water by physical retention based on pore size exclusion, most commonly using hollow fiber ultrafilters (Hill et al., 2007; Morales-Morales et al., 2003). Some hollow fiber filters are very efficient and are considerably less expensive than the 1-MDS filters. However, equipment setup for ultrafiltration is still not readily portable, thus limiting its field application. Furthermore, the large volume of buffer required for elution of both cartridge adsorbent filters and ultrafilters is not effective for sensitive PCR analysis, as only a small percentage of the concentrated sample can be analyzed in an individual amplification reaction. Glass wool filters and electropositive nanoalumina fiber filters are two types of adsorbent filters able to concentrate multiple types of viruses in water (Lambertini et al., 2008; Li et al., 2010). While both filters are cost effective, the former has highly variable recoveries, and the latter has not been tested for recovering viruses in turbid water. Both require relatively large elution volumes.

Because of its low cost ($1 USD) and the simplicity of the method, the electronegative 0.45µm pore size cellulosic filters such as type HA (Millipore, Inc., Bedford, MA) remain commonly used. Combined with PCR, this type of filter has been successfully applied for monitoring the presence of enteric viruses in environmental waters (Fuhrman et al., 2005). However, given its small pore size, the filtration rate can be very slow when filtering turbid water. Early studies proposed the use of prefilters (for example, fiber glass) or additional larger diameter membrane filters to increase the flow rate (Farrah et al., 1976; Metcalf et al., 1974), but neither approach is convenient or effective.

To improve the flow rate and reduce filtration time when analyzing water samples with high turbidity, a large pore size SCWP filter (8.0 µm pore size, 47 mm diameter) may be a useful alternative to concentrate viruses from source water samples. Because it was reported that a large pore filter (5.0 µm pore size, 47 mm diameter) was effective for recovering poliovirus in water (Haramoto et al., 2004) and the SCWP filter is made of the same materials as the HAWP filter, mixed-cellulose esters, we hypothesize that the replacement of the HAWP filter with the SCWP filter will significantly reduce the filtration time and have a comparable recovery of viruses in turbid water without any other modification. In this study, we evaluated the efficiency of the large pore size SCWP filters to concentrate adenovirus 41 in source waters in comparison to HAWP filters, and also developed a simple reconcentration method based on evaporation in preparation for real-time PCR analysis.

5.2 Materials and methods

5.2.1 Water samples

Source water samples used for seeding experiments during methods optimization were collected from the Orange Water and Sewer Authority (OWASA), Carrboro, North Carolina. Water quality parameters of samples, including pH, turbidity, manganese (Mn), total organic carbon (TOC) and dissolved organic carbon (DOC), were measured according to Standard Methods (2000).

5.2.2 Primary concentration

Each one liter source water sample was adjusted to pH 3.5, then spiked with 0.1 mL of adenovirus 41 (ATCC VR-930, available from the American Type Culture Collection, ATCC) working stock (to achieve approximately 2.5×10^6 virus particle/L). The samples were initially shaken by hand for 1 minute and the viruses and water were allowed to interact for 30 minutes. Then 1 L of virus containing water sample was gradually poured into a filtration system consisting of a 300-mL funnel and a 1-L glass base (The system is the same as described for enumeration of *E. coli* and Enterococci in water using EPA Method 1600 and 1603, respectively). Both the SCWP filter (8.0 µm pore size, 47 mm diameter) and HAWP filter (0.45 µm pore size, 47 mm diameter) were tested for the recovery for adenovirus 41. Filtration was started with the assistance of gentle vacuum (<150 mm Hg) and then negative pressure was increased when water flow slowed. After the water sample completely passed through the filter, the filter was put into a 50-mL polypropylene centrifuge tube with 4 mL of eluting buffer, and the viruses were eluted from the filter by vortex mixing the eluting buffer for 2 minutes. The eluting buffer was made using 1.5% beef extract plus 0.75% glycerol (pH=9.0). Then the viruses from 0.1mL of this primary filter eluate were detected by the real-time PCR assay for measuring the virus recovery of this step.

To optimize the performance of the SCWP filter, experimental conditions of water pH, magnesium ion concentration and eluting buffer were tested. The pH of water samples was adjusted to either 2.5, 3.5, 4.5 or 7.5 prior to filtration. Because magnesium ions in water might improve virus recovery using negatively charged membranes, we tested the effects of adding $MgCl_2$ to water samples prior to filtration. This step is also included in Standard Methods (2000). Briefly, 10 mL of 10 M $MgCl_2$ was added to 1 L water samples, and then the pH was adjusted to 3.5. After filtration, the membrane was rinsed using 25 mL of 0.14M NaCl (pH=3.5) to remove the extra magnesium. Finally, in addition to 1.5% beef extract (BBL, Benton Dickenson and Company, Sparks, MD) plus 0.75% glycerol, we also tested two frequently used eluting buffers: 3% beef extract (pH=9.0-9.3) and 1mM NaOH (pH=10.8-10.9).

To determine if the large pore size filter overcomes the influence of water turbidity, we compared the SCWP filter with the HAWP filter in water having different turbidities. Source water samples were serially diluted to a turbidity of 4.0, 3.0, 2.0 and 1.0 NTU. The filtration time was recorded and virus recovery was measured using the PCR method described below.

5.2.3 Reconcentration

We tested a novel reconcentration method using evaporation and compared it to the Centricon-YM100 ultrafiltration (MWCO: 100,000, Millipore Inc., Medford, MA) method. Four milliliters of the primary eluate of source water was split into two samples for reconcentration. One was reconcentrated using evaporation and the other was subjected to ultrafiltration. For evaporation, 2 mL of the primary concentrate was mixed with 20 µL of 1% antifoam solution (Sigma-Aldrich, Inc., St. Louis, MO). The mixture

was transferred into a 50-mL polypropylene centrifuge tube and then the sample was heated in a waterbath at 55°C. A soft hose connected to a l-mL micropipette tip was used to introduce air flow (about 1-5 bubbles per second) into the sample. When the sample was close to dry (0-0.1 mL of sample remaining), 0.1 mL of PBS buffer (pH=7.4) was added into the tube to resuspend the viruses. For ultrafiltration, 2 mL of the primary concentrate was filtered by the Centricon-YM100 ultrafilters and then the viruses were eluted using 0.1 mL of PBS buffer. The filtration and elution were conducted with the assistance of centrifugation following instructions from the ultrafilter supplier. Speed and time of centrifugation were dependent on the volume and the turbidity of the samples.

Potential aerosolization and cross contamination during evaporation was also tested. To examine whether viruses could be released into the air during evaporation, 0.2 mL of adenovirus 41 from the working stock was spiked into 2 mL of source water samples in a 50-mL polypropylene centrifuge tube. Then the tube was placed into a waterbath at 55°C and air was blown into the sample. A cap was put on the top of the tube to collect evaporated water at 2 and 4 hours, respectively. The adenovirus was analyzed from 0.1 mL of the evaporated water sample. To examine whether air exposure could contaminate the water samples, three tubes containing 2 mL of PBS buffer were opened to air for a few hours (2-6 hrs). The samples from these tubes were evaluated for the presence of adenoviruses by PCR.

5.2.4 Detection of adenovirus 41 using real time PCR

Adenovirus 41 was detected using a Taqman real-time polymerase chain reaction (PCR) method (Jothikumar et al., 2005). First, the viral nucleic acid was extracted and purified using guanidinium thiocyanate (GuSCN) lysis buffer (Jothikumar et al., 2010) and silica

microcolumns. Briefly, 100 µL of GuSCN lysis buffer was added into 100 µL of the samples, and then the mixture was incubated at room temperature. After 10 minutes, 200 µL of 100% ethanol (RNase-free) was added to the mixture and vortex mixed for 5 seconds. The total solution (400 µL) was transferred to a high bind RNA mini column (OMEGA BIOTEK, Inc., Norcross, Georgia, USA) and centrifuged for 1 minute at 14,000x g. The column was washed using 500 µL of 70% ethanol and then centrifuged for 1 minute at 14,000x g. After the column was twice washed using 70% ethanol, viral DNA was eluted from the column using 50 µL of RNase-free water and centrifuged at 14,000x g for 1 minute. The extracted DNA was transferred to a 500 µL Eppendorf tube for immediate PCR quantification or stored at -80°C. The following primer sets and probe (Jothikumar et al., 2005) were used to amplify the fiber gene of the adenovirus 41 genome: forward primer JTVFF (AACTTTCTCTCTTAATAGACGCC), reverse primer JTVFR (AGGGGGCTAGA AAACAAAA) and the probe JTVFAP (GCGAAGAGTGCCCGTGTCAG). The QuantiTect probe PCR kit (QIAGEN, CA) was used in the real-time PCR system. The reaction mixtures contained 2 µL of template DNA or sample extract, 0.5 µL of 50 µM primers, 0.5 µL of 5 µM fluorogenic probe, 12.5 µL 2X quantitech PCR master mix and 9 µL RNase-free water to a final volume of 25 µL. The PCR amplification was performed with the following conditions: denaturation at 95 °C for 15 minutes, followed by 45 cycles of denaturation at 95 °C for 10 seconds, annealing at 55°C for 30 seconds, and elongation at 72 °C for 15 seconds. All reactions were conducted in duplicate. The real-time PCR reaction was carried out in a Smart Cycler Thermocycler (Version 2.0c; Cepheid, Sunnyvale, CA, USA). The concentration of virus was calculated based on a standard curve, which was generated by

detecting the virus in a series of dilutions of the stock. The viruses in eluting buffer were diluted 5 fold (0.2 mL of virus containing eluting buffer + 0.8 mL PBS buffer) and measured by real-time PCR using the standard curve. To measure the number of seeded viruses, a reference control was set in each trial, which was made by adding 0.1 mL of adenovirus 41 from working stock directly to 4 mL of eluting buffer. Autoclaved PBS buffer was used as a negative control for the PCR assay. PBS buffer was also used as an external control to evaluate the potential PCR inhibition effects of concentrated source water samples and the eluting buffer. To evaluate the PCR inhibition effects of the eluting buffer, 0.1mL of adenovirus 41 from the working stock was added into 4 mL of the eluting buffer and 4 mL of PBS buffer, respectively. Then viruses in both buffers were detected by the real-time PCR assay. If there is no significant difference in the virus concentrations of both buffers measured by the PCR assay, it is assumed there are no PCR inhibitors in the eluting buffer. The same strategy was used to evaluate the PCR inhibition effects of source water samples. 1 L of the blank source water and 1 L of PBS buffer were filtered through the SCWP filter and eluted by 4 mL of the eluting buffer, respectively. Then both elutates were seeded with 0.1mL of adenovirus 41 working stock. If the measured virus concentrations have no significant difference, it is assumed that there are no PCR inhibitors in the source water sample.

5.2.5 Virus recovery calculation and statistical analysis

Percent recovery of adenoviruses was calculated using the following equation: percent recovery = the number of recovered viruses/ the number of seeded viruses*100. The Wilcoxon-Mann-Whitney (WMW) test and the Kruskal Wallis (KW) test were used to examine the difference of the mean recoveries of adenovirus 41 at different conditions.

The Wilcoxon-Mann-Whitney test is a non-parametric version of the independent samples t-test and the Kruskal Wallis test is a non-parametric counterpart to ANOVA (Analysis of Variance). Both tests do not require a normal distribution of the data of dependent variables. Here, the dependent variable is the percent recovery, and the categorical independent variables (factors) include water pH (4 categories), magnesium (2 categories), eluting buffer (3 categories), types of filter (2 categories) and water turbidity (4 categories). All statistical analyses were carried out with SAS 9.2 software (SAS Inc., Cary, NC) and the level of significance was set at 0.05 for all tests.

5.3 Results

5.3.1 Water quality of samples for seeding experiments

All source water samples used for seeding experiments were collected from the OWASA, NC water treatment plant. On average, pH and turbidity were 6.93 and 5.04 NTU, respectively (Table 5.1). Average concentrations of TOC and DOC were 4.94 and 5.07 mg/L, respectively. *E. coli* density was very low, ranging from 1 to 6 MPN/100 mL. No adenovirus 40/41 were detected in these samples.

5.3.2 Optimization of the recovery of the large pore size filter

We measured the recovery of the large pore size filter for adenovirus 41 from 1 L volumes of source water under different conditions. Using 1.5% beef extract plus 0.75% glycerol as eluting buffer, the recovery was approximately 55±19% (mean ± standard deviation) when water pH was 3.5, and was slightly lower when water pH was 2.5 ($n=13$, $p=0.626$, WMW test). However, the recovery was less than 5% when water pH was 4.5 or above and significantly lower ($n=32$, $p<0.001$, KW test) (Figure 5.1). The addition of magnesium (0.1 M) in the water sample slightly improved the recovery ($n=14$, $p=0.478$,

WMW test). We also compared the recoveries obtained by different eluting buffers. Virus recoveries using 3% beef extract, 1 mM NaOH and 1.5% beef extract plus 0.75% glycerol were 30%, 40% and 55%, respectively, with the highest recovery achieved using 1.5% beef extract plus 0.75% glycerol (n=17, p=0.001, KW test) (Figure 5.2).

5.3.3 Comparison of SCWP filters and HAWP filters

Turbidity is a major factor that hinders the application of negatively charged membrane filters because particles can clog the filter and slow or stop the filtration process. For this study filtration time was compared for two types of filters using 1 L of serially diluted water samples (Figure 5.3). The SCWP filter was significantly more efficient than the HAWP filter (n=24, p=0.003, WMW test), reducing the filtration time up to 85%. Filtration using the HAWP filter had a higher recovery than that measured using the SCWP filter (Figure 5.4). The difference in the recoveries ranged from 7% to 18%. The results of the statistical test indicated the percent recovery of adenovirus 41 was not significantly different for the different filter types (n=31, p=0.060 WMW test). Turbidity was also not found to significantly affect recovery efficiency (n=31, p=0.398, KW test).

5.3.4 Reconcentration of adenoviruses 41 by evaporation

We developed a simple method to reconcentrate viruses by evaporation. As shown in Table 5.2, the evaporation method achieved approximately 86.8% adenovirus 41 recovery in the primary filter eluate concentrate of 1 L of source water. It took about 1 hr to evaporate samples from 2 mL to 0.1mL or dryness. We also compared this method with a filtration method using Centricon ultrafilters, which retain viruses based on molecular weight cut off (MWCO) and are commonly used to reconcentrate viruses in water samples. The filtration method obtained only 35.3% recovery of adenovirus 41

from the primary filter eluate concentrate of source water, which was significantly lower than that obtained using the evaporation method (n=15, p=0.02, WMW test). In addition, it took 90 minutes or longer. We also tested the potential for virus aerosolization and cross contamination during evaporation. The results indicated that no viruses were released into air and no viruses from air entered the samples (data not shown).

5.4 Discussion

In this study, we used a large pore size (8.0 µm) cellulose ester membrane filter to concentrate human adenoviruses from 1 L water samples. This procedure required 85% less process time compared to the traditional method using 0.45 µm pore size filter to concentrate viruses in source water. In addition, evaporation was applied to reconcentrate viruses in the filter eluates, which achieved approximately 86.8% recovery at no materials cost. To our knowledge, this is the first study using large pore size filters to concentrate adenoviruses and the first study using evaporation to reconcentrate viruses in small volumes of water samples.

Negatively charged cellulose membrane filters are widely applied to concentrate viruses in water samples because the method is simple and cost-effective. The membrane filter carries negative charges, which can adsorb positively charged viruses when water pH is below the isoelectric point of the virus. In this study, the recovery of viruses from the large pore size filters was highest when water pH was 3.5, consistent with previous studies using HA series filters and the recommendation of Standard Methods (2000). The recovery was very low when water pH was 4.5 or above, perhaps because the extent of positive charge of the virion is not great enough to facilitate adsorption. According to Favier et al (2004), the isoelectric point (pI values) of the major virion surface proteins of

hexon, penton base, long fiber (head) and short fiber (head) were predicted to be 5.49, 5.75, 7.51, and 9.31, respectively. Therefore, it is likely that all virus adsorption sites to surfaces such as membrane filters will be electropositive to varying magnitudes at pH levels below pH 4.5. For the negatively-charged filtration method, a key step is to adjust water pH. It is easy to operate when the volume of water samples is small, for example, 1 to 2 L. However, adjusting water pH can be tedious for a large volume of water samples, such as 10 L or 100L. In this situation, other filtration methods, such as the cation-coated filtration method (Haramoto et al., 2004) or the positively charged filtration method (Sobsey and Jones 1979), might be more appropriate. In previous studies, bivalent or trivalent salts (eg. $MgCl_2$, $AlCl_3$) were found to promote virus adsorption to microporous filters (Gerba 1984; Walllis et al., 1979). This interaction might be the result of direct effects, for example, by promoting hydrophilic ionic interactions, and of indirect effects, for example, by changing water pH or forming flocs (Lukasik et al., 2000). However, the ability of cations to increase adsorption also depends on the types of viruses and filters tested (Lukasik et al., 2000). Our results showed that the addition of $MgCl_2$ improved virus recovery, but the improvement was not statistically significant.

Eluting buffers also influence the recovery of viruses from negatively charged filters. Traditionally, 3% beef extract has been used as an eluting buffer not only because the organic compounds in 3% beef extract play an important role in the elution of viruses by hydrophilic and hydrophobic interactions, but also because it can facilitate the subsequent reconcentration by bioflocculation (Katzenelson et al., 1976; Landry et al., 1978). Recently, 1 mM NaOH (pH= 10.5-10.8) was recommended as an eluting buffer and applied in a number of studies (Haramoto et al., 2005; Li et al., 2010). The use of high

pH buffers for elution of viruses adsorbed to filters has been widely practiced for several decades (Sobsey 1976). These previous studies showed that 1 mM NaOH obtained a better recovery than 3% beef extract, which might have resulted from potential PCR inhibition effects of organic compounds in the beef extract. Our study also showed that 1 mM NaOH was a better buffer than 3% beef extract in terms of virus recovery. However, we found 1.5% beef extract with 0.75% glycerol (pH= 9.0) achieved an even better recovery than 1 mM NaOH. A plausible reason is that we used a small volume (4 mL) of eluting buffer, which cannot immerse the entire filter. The 1.5% beef extract buffer with glycerol can temporarily form foams, thus increasing its bulk volume and perhaps washing the filter more thoroughly. Furthermore, glycerol is known to stabilize protein structure and capsid proteins are important to the integrity and function of viruses. Protein structure and function are sensitive to solvent viscosity, dielectric constant, and pH, as well as to subtle, cosolvent specific thermodynamic effects, while glycerol is perhaps the most widely used viscogenic cosolvent with water, which makes possible large changes in solvent viscosity with relatively small changes of pH and dielectric constant (Gekko and Timasheff 1981; Gekko 1982). In addition, our experiments showed there was no PCR inhibition caused by this buffer.

The filtration time and recovery of the large pore size filters (SCWP) and the small pore size filters (HAWP) were compared (Figure 5.3 and Figure 5.4). Remarkably, the SCWP filters have a much faster flow rate than the HAWP filters for filtration of 1 L water samples across a range of turbidity, which saved up to 85% of filtration time. When water turbidity is above 2 NTU, using HAWP filters can be very time-consuming, taking 3-12 hours to filter 1L of water. However, using a SCWP filter, the filtration can be finished

in 1 or 2 hours. Both types of filters can recover ≥ 35% of adenovirus 41 present in water samples at different turbidities. The recovery of HAWP filters was higher than that of SCWP filters, which could be expected due to virion adsorption decreases with flow rate increases (Scutt 1971) and adsorptive area of filters decreases with pore size increases.

Reconcentration of viruses is commonly needed because the concentration of viruses in natural water samples is very low. Some simple and cost-effective reconcentration methods were developed in earlier studies, such as organic flocculation (Katzenelson et al., 1976) and polyethylene glycol precipitation (PEG) (Lewis and Metcalf 1988). However, these methods have some disadvantages, such as varying and sometimes low recovery among different viruses, long processing times, and the inability to optimize concentration conditions for different viruses (Lewis and Metcalf 1988). Recently, some specifically designed ultrafilters were applied to reconcentrate viruses, including Centriprep ultrafilters (Haramoto et al., 2004), Centricon-series ultrafilters (Hill et al. 2007) and Amicon ultrafilters (Li et al., 2010) made by Millipore Inc., which cost about $4 USD per filter. These filters retain viruses based on their MWCO. We selected the Centricon YM100 filter because it has a molecular weight cutoff (MWCO) of 100,000 Dalton, and theoretically, it can be effective to retain adenoviruses, whose molecular weights range 2,000,000-2,500,000 Dalton (Green et al., 1967). Other Centricon-series ultrafilters with smaller MWCOs can also be used to retain adenoviruses. However, they are more likely to get clogged, thus will take a longer time to filter water samples. In this study, we developed a method to reconcentrate viruses by evaporation. With the assistance of heating and air flow, 2 mL of primary concentrate can be dry in 1-1.5 hours. In theory, the recovery will be 100% and in our tests, recovery was 86.8% for source

water. In contrast, using the Centricon-YM100 filters, we only obtained 35.3% recovery from the filter eluate concentrates of source water. In addition, water turbidity does not affect reconcentration time for evaporation but can be a factor for ultrafiltration using ultrafilters. Furthermore, the evaporation method has no to low supply costs and is easy to perform. One concern of evaporation is the potential for aerosolization or sample contamination during the evaporation process. The results of our experiments to investigate this possibility suggested this would be unlikely, at least for adenovirus 41. To further ensure no contamination, some measures could be easily taken, for example, putting some cotton or other filter barrier materials on the top of the tube, or putting the tube into a closed chamber. Although evaporation is new for concentrating viruses in water, it is a common method used to concentrate chemicals in water and some well-designed devices are commercially available, such as the rotary vacuum-evaporation apparatus or the microwave-assisted evaporator (Link and Kingston 2000; Privalova and Prokopenko 1969). Our study revealed that the evaporation method has effective performance for concentrating viruses. Therefore, there is a market potential for manufacturers to design and produce devices specific to concentrate viruses using this process. It should be pointed out that heating at 55-60 °C may inactivate adenoviruses and other viruses, thus this reconcentration step is not recommended when cell culture-based methods are planned for subsequent virus detection.

Combining the primary cellulose ester filter adsorption-elution concentration method and the evaporation reconcentration method, we greatly improved the detection limit of the real-time PCR assay. Our primary step concentrated viruses 250-fold with 50% recovery, and the reconcentration step concentrated viruses another 20-fold with 86.8% recovery.

Therefore, we concentrated samples 5000 fold with total recovery above 40% of initial viruses. If we assume that the PCR assay can detect 1 virus particle in each PCR reaction tube (containing 2µL DNA extract), the lowest limit of detection for this assay is approximately 6 viral particles per 100 mL of water. If multiple PCR reactions tubes were used, the detection limit would be even lower.

Currently, microbial water quality monitoring and assessment are largely based on the measurement of several microbial indicators, which in part is because the direct detection of viral pathogens is commonly time-consuming, costly and technically difficult. Our concentration method is very simple and cost-effective (total expendable materials cost of ~$1 USD), which might prompt the advancement of routine monitoring of viral pathogens. In addition, this method can concentrate 1L of source water samples 5000 times within 2 hours. With the addition of time used for virus detection by real-time PCR, we can obtain results for viral analysis in 5 or 6 hours by these methods. Rapid methods for evaluation of recreational water and drinking water are sorely needed. For example, the U.S. Beach Protection Act of 2007 requires the development of rapid testing methods that can provide water quality data within hours of sampling. These methods are needed to help managers decide whether to post or close a beach within 24 hours. Our method would allow results within hours of sampling. In addition, the results obtained with the SCWP filter in source water suggest that this method is capable of detecting viruses in samples with high turbidity.

Table 5.1 Water quality of source water samples for seeding experiments

Parameters	mean ± STD	Parameters	mean ± STD
pH	6.69±0.27	TOC (mg/L)	4.94±1.27
Turbidity (NTU)	5.04±1.83	DOC (mg/L)	5.07±0.39
Manganese (mg/L)	0.20±0.16	*E. coli* (MPN/100mL)	3.67±2.52

Table 5.2 Comparison of evaporation and filtration using Centricon filters to reconcentrate adenovirus 41

Methods	Evaporation	Filtration (Centricon filter)
Method description	As water is evaporated with the assistance of heating and air flow, viruses remain in the tube	As water is passed through the filter, viruses are retained during centrifugation.
Processing time (source water)	60-90 minutes	90 minutes or longer
Concentration factor	20-fold	20-fold
Recovery(source water)	86.8±18.7%	35.3±9.6%
Cost of materials	$0 USD	$4 USD
Difficulty of the method	Simple	Relatively complex
Limitations	May not be applicable for large sample volumes	May not be applicable for samples with high turbidity

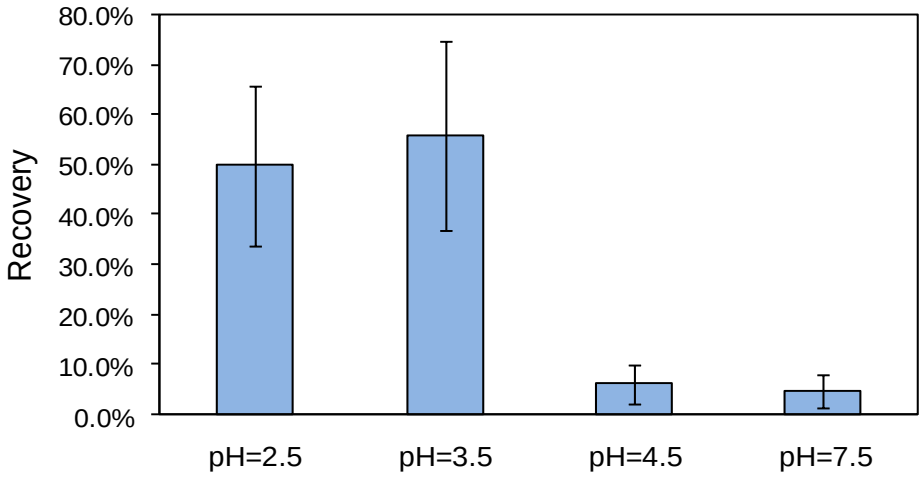


Figure 5.1 The effect of water pH on the recovery of the SCWP filter for adenovirus 41 in source water (eluted by 1.5% beef extract plus 0.75% glycerol). The mean recoveries are significantly different when water samples were adjusted to different pH (n=32, $p<0.001$, KW test). However, mean recoveries were not significantly different between pH 2.5 and pH 3.5 (n=13, $p=0.626$, WMW test)

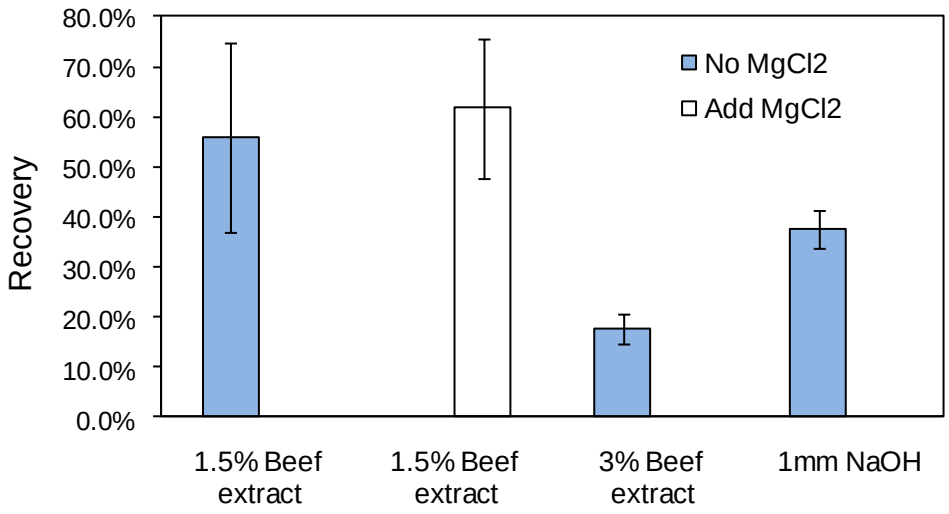


Figure 5.2 The effects of magnesium and eluting buffers on the recovery of the SCWP filter for adenovirus 41 in source water (pH=3.5). Data labeled 1.5% beef extract represent an elution buffer of 1.5% beef extract containing 0.75% glycerol. The mean recoveries are significantly different when different eluting buffers are used (n=17, p=0.001, KW test) but not significantly different whether $MgCl_2$ was added or not (n=14, p=0.478, WMW test)

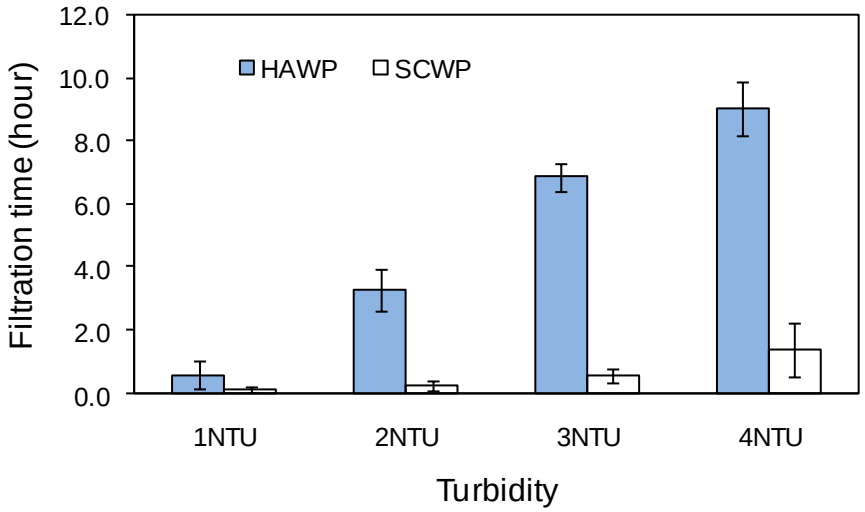


Figure 5.3 Comparison of the filtration time by the HAWP filter and the SCWP filter for water samples at different turbidities. The filtration time was significantly different between HAWP filters and SCWP filters (n=24, p=0.003, WMW test) and also significantly different at different water turbidities (n=24, p=0.03, KW test)

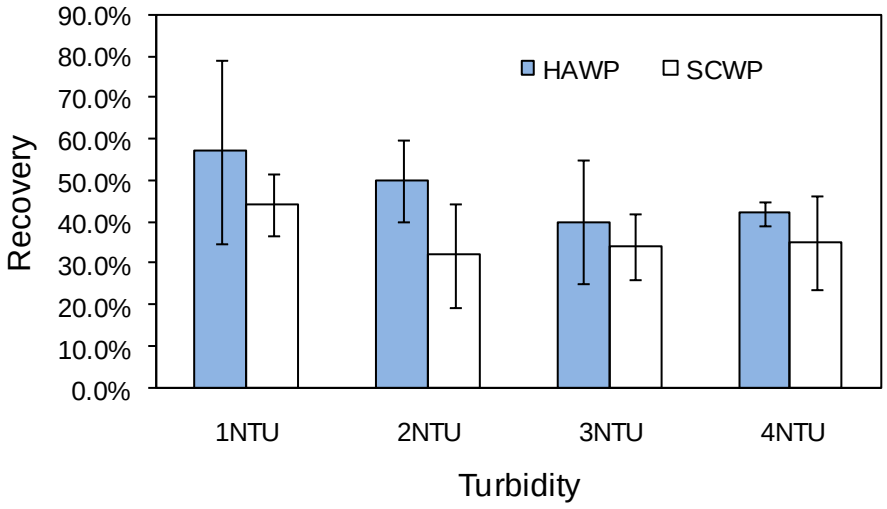


Figure 5.4 Comparison of the recovery of the HAWP filter and the SCWP filter for adenovirus 41 in water samples at different turbidities. The mean recoveries were not significantly different between HAWP filters and SCWP (n=31, p=0.060, WMW test) and also not significantly different at different water turbidities (n=31, p=0.398, KW test)

Chapter 6

EFFICACY OF HOLLOW FIBER ULTRAFILTER FOR RECOVERING MULTIPLE MICROORGANISMS IN WATER

Abstract

Ultrafiltration as an effective, rapid and affordable technique has been increasingly applied to recover multiple classes of microbes in water. However, the performance of ultrafilters is influenced by many factors, including the quality of water samples and the different types of microbes. In this study, two types of hollow fiber ultrafilters were tested to evaluate their ability to recover 5 types of microbes in both source and finished drinking water. The influences of filtration setup, pH and clay particles on the recovery were also evaluated. The results revealed that the hollow fiber ultrafilters efficiently recovered *E. coli* KO11, *E. coli* O157:H7 and bacteriophage MS2, but recovery rates of *Bacillus atrophaeus* spores and adenovirus 41 were remarkably different between source water and finished water. The change of pH from 6.5 to 8.5 increased the recovery rates for adenovirus 41 in source water but had no impact on other microbes. The endcap modification of filters reduced filtration processing time drastically, especially when filters were applied in parallel, without affecting recovery efficiencies. Our study suggested that types of microbes and water matrix are two major influencing factors of concern in the application of hollow fiber ultrafilters.

6.1. Introduction

Waterborne pathogens, including bacteria, viruses and parasites, pose a risk to public health when people are exposed to contaminated drinking water and recreational water. Since pathogens in water are usually in low concentrations, detection and quantification of pathogens require concentration and recovery of microorganisms as a primary step. In response to routine microbial quality monitoring or terrorism incidents, it is crucial to recover multiple classes of microbes rapidly and simultaneously. To date, a variety of methods have been developed to concentrate specific microorganisms, such as continuous flow centrifugation for bacteria and protozoa (Swales and Wright, 2000; Borchardt and Spencer 2002), and immune-affinity concentration, ionically charged filtration and ionically charge modified filtration for enteric viruses (Schwab, et.al., 1996, Sobsey and Glass 1980; Simmons 1995). However, these techniques, to some extent, are time-consuming, expensive, technically-difficult, or ineffective for multiple classes of microbes.

An alternative technique to concentrate multiple classes of microbes is ultrafitration. Ultrafilters typically have pore sizes in the range of 0.01-0.10 µm. Because of their small pore sizes, ultrafilters are able to concentrate multiple classes of microbes simultaneously. The method based on tangential flow hollow-fiber ultrafiltration (HFUF) has been successfully used for concentrating *Cryptosporidium* oocysts (Simmons et al., 2001; Francy et al., 2004), bacteria, viruses and parasites (Juliano and Sobsey 1998; Morales-Morales et al., 2003; Hill et al., 2005; Rajal et al., 2007) in drinking, surface and rain water samples. A number of studies were conducted to improve this method by using different elution buffers, pretreating the filter, or increasing flow rate to reduce process

time (Hill et al., 2005; Simmons 2007; Anderson 2008). The detailed procedures using HFUF have been published (Lindquist et al., 2007; Polacyk et al., 2008).

According to the previous studies, recoveries using hollow fiber ultrafilters have high variability. As shown in Table 6.1, recovery rates have a large variability for different microbes in different studies, the same microbes in the different studies, and even the same microbes in the same studies. It is hypothesized that microbe type, water matrix, filter configuration, seeding level and other factors might affect recovery efficiencies. To date, few studies have specifically evaluated the performance of hollow fiber ultrafilter under the influence of multiple factors.

The objectives of this study are: (1) to design a rapid HFUF method to recover both microbial indicators (*E coli* KO11, bacteriophage MS2 and *Bacillus atrophaeus* spores) and pathogens (*E. coli* O157:H7 and adenovirus 41) from water; (2) to analyze the potential influences of microbe type, water matrix, seeding level and filtration setup on the performance of filters, and (3) to evaluate impacts of water pH on the recovery of filters.

6.2 Materials and methods

6.2.1 Water samples collection and water quality analysis

Water samples were collected from the Orange County Water and Sewer Authority (OWASA), Carrboro, North Carolina, from May 2008 to June 2009. Both source (untreated) and finished (treated) drinking water samples were collected in 10 L Cubitainer. The source water originated from either University Lake or Cane Creek reservoir, NC, USA, both of which are protected surface water impoundments. Finished water samples were dechlorinated by adding 1 g of sodium thiosulfate per 10 L before

spiking microbes. The removal of chlorine residual in water was verified using the DPD (N, N-diethyl- p- phenylendiamine) method (American Public Health Association et al., 2000).

Physical and chemical water quality parameters of the samples, including color, pH, iron, manganese (Mn), fluoride, turbidity, alkalinity (ALK), hardness, total organic carbon (TOC) and dissolved organic carbon (DOC), were measured. Color was measured by Hach turbidimeter, Model 2100 AN (HACH Company) using HACH APHA Platinum-Cobalt Standard Method; iron was measured by Hach DR500 Spectrophotometer using Hach Method 8008 and FerroVerr Method; manganese was measured by Hach DR500 Spectrophotometer using 1-(2-Pyridylazo)-2-Naphthol PAN Method (Hach Method 8149); floride, turbidity, pH and DOC were measured by Accumet AR25 pH/MV/ISE bench top meter, HACH 2100AN Turbidimeter, Accumet AR15 pH/MV bench top meter, respectively, following the standard methods (American Public Health Association et al., 2000). Alkalinity was measured by Bromocresol Green-Methyl Red Indicator. TOC and DOC were measured by Phoenix 8000 using Persulfate-Ultraviolet Oxidation Method. Hardness was measured by EDTA Titrimetric Method derived from Standard Methods (American Public Health Association et al., 2000).

6.2.2 Microorganisms and seeding experiments

A suit of microbes, including 3 microbial indicators (*Escherichia coli* KO11, bacteriophage MS2, *Bacillus atrophaeus* spores) and 2 pathogens (*Escherichia coli* O157:H7 and adenovirus 41), were tested in this study. *Escherichia coli* KO11 (ATCC 55124), bacteriophage MS2 (ATCC 15597-B1) and adenovirus 41 (ATCC VR-930), were available from the American Type Culture Collection (ATCC). *E. coli* O157:H7

was obtained from BTF Precise Microbiology, Inc. (Pittsburgh, PA), and *Bacillus atrophaeus* spores stock was purchased from SGM Biotech, Inc. (Bozeman, MT).

The five test microorganisms were simultaneously spiked in water. Before spiking, *E. coli* KO11 and *E. coli* O157:H7 were cultured overnight using tryptic soy broth (TSB) at 35°C. The TSB growth medium for *E. coli* KO11 was supplemented with chloramphenicol (40 mg/L). These microorganism stocks were diluted and mixed in 100 mL of PBS (Phosphate buffered saline) solution (pH=7.2). Then the mixtures were spiked into the dechlorinated finished water samples or directly spiked into the source water samples. The seeded water samples were agitated by a magnetic stir bar for a half hour. Fifty milliliters of the seeded water sample were collected as the initial sample, in which the concentration of microbes was measured. A variety of seeding levels were tested, which ranged from 10^5 to 10^9 cfu or pfu per liter of water.

6.2.3 Filtration process

Two types of commercial hollow-fiber ultrafiters, Hemoflow F80A and Optiflux® F200A, were evaluated (Fresenius Medical Care, Lexington, MA). The Hemoflow F80A polysulfone dialysis filter has a parallel flow through the hollow fibers with a MWCO (molecular weight cut off) of 15,000 to 20,000, a surface area of 1.8 m^2, and a fiber inner diameter of 200 μm, while the Fresenius Optiflux® F200A filter has a more turgid flow with a greater surface area. To reduce processing time, the inlet and outlet openings of ultrafilters were modified by computer-designed stainless steel endcaps instead of the original endcaps (Anderson 2008). The setup of filtration encompassed two models, the normal model and the parallel model (Figure 6.1). In the normal model, the inlet of filters was connected to a pressure gauge and the tubing of a peristaltic pump (Burt Process

Equipment, Inc. Hamden, CT Masterflex I/P Precision Brushless Drive) fitted with Masterflex tubing having ½' internal diameter. The tubings of the inlet and the outlet of the filter were connected by a T shape connector, by which the tubing was connected to the water sample in the Cubitainer. In contrast, the parallel model was comprised of two filters connected in parallel, each filter was connected with a pressure gauge and a peristaltic pump as described above. The peristaltic pump was started to recirculate water samples at low pressure (5 psi) to ensure no leaks, and then pressure was gradually increased to 15 psi (the maximum pressure for this system is up to 25 psi). The recirculation of water was when the Cubitainer was emptied of water leaving some residual water in the tubing and the filter housing. The eluting solution (300 ml) was recirculated in the filtration system at low pressure (5 psi) to resuspend microbes possibly retained in the filter. The eluting solution consists of 10g/L of Laureth-12 and 50 µl/L of antifoam-A in PBS (Phosphate buffered saline) solution. The eluate was collected as the final concentrated sample. Prior to experiments, pressure gauges were sterilized using 70% alcohol, other stuffs and solutions that have potentials to microbially contaminate water samples were autoclaved.

6.2.4 Microorganism assays

E. coli KO11 and *E. coli* O157:H7 were assayed by spread plate method. *E. coli* KO11 was detected using tryptic soy agar (TSA) plus 40 µg/mL of chloramphenicol (MacConkey agar, a selective agar for Gram-negative bacilli, is also good for detecting *E. coli* KO11. Since the results from both media were similar, here only TSA was used). *E. coli* O157:H7 was detected by MacConkey Agar II with Sorbitol. MS2 was assayed using the single agar layer plaque (SAL) method with *E. coli* C3000 in log-phase as host

(USEPA 2001). *Bacillus atropheaus* spores were quantified using AK2 agar after the samples were heated at 80℃ for 10 minutes. All the plates were incubated at 35℃ in inversed plates. The microbial colonies or phages were counted after 16-20 hours.

The procedures for detection of adenovirus 41 using real-time PCR were described in Chapter 5.

The virus in PBS solution was used as an external control to evaluate the effects of PCR inhibition during the detection of adenovirus 41. Specifically, 300 mL of adenovirus 41 stock in PBS buffer was split in 3 aliquots. One aliquot was spiked into 10L of source water, another was spiked into 10 L of finished water, and the remaining was diluted 100-fold in PBS buffer as an external control. Theoretically, the concentrations of the virus were same in the source water sample, the finished water sample and the external control. PCR inhibition was indicated if there was a significant decrease of concentration of virus in water samples in contrast to the virus in external control.

The samples showing PCR inhibition were pretreated by chloroform extraction to reduce inhibition effects. Briefly, the same volume of chloroform was added into the samples. After shaking for 1 minute, the mixture was centrifuged at 3000 rpm, 30 minutes and 4℃. The supernatant was collected, from which the viral nucleic acid was extracted by the same procedures described above.

6.2.5 Effects of water pH

To evaluate the pH effects, the pH values of water samples were adjusted to 6.5, 7.5 and 8.5, respectively. The water samples were filtered by using a F80A filter in the normal setup (a single filter model) and the microbes were measured following the same procedures described above.

6.2. 6 Recovery calculation and statistical analysis

Percent recovery of the filters was calculated by the following equation:

Recovery (%) $= \frac{C_F \times V_F}{C_I \times V_I} \times 100\%$. Where, C_F is the concentration of microbes in the final concentrated sample, V_F is the volume of the final concentrated sample, C_I is the concentration of microbes in the initial sample, and V_I is the volume of the initial sample, which is 10 L in this study.

One-way ANOVA was used to test the difference of water quality parameters between source water and finished water. The multi-way ANOVA analysis was conducted to examine the effects of multiple factors on the microbe recovery of filters, including water type, filter type, filtration setup, microbe type and microbe seeding levels. Microbial concentrations (e.g., seeding levels) were log transformed before analysis. All analyses were conducted using SPSS 16.0 software (SPSS, Inc., Chicago, Illinois).

6.3 Results

6.3.1 Water quality

Water quality of each sample was measured before a filtration experiment (Table 6.2). In source water, turbidity, TOC, DOC, Mn, and iron were 3.13 NTU, 7.14mg/L, 4.87mg/L, 0.157mg/L and 0.46 mg/L on average, respectively. In finished water, turbidity, TOC, DOC, Mn, and iron were 0.08 NTU, 2.42 mg/L, 2.34 mg/L, 0.004 mg/L and 0.005 mg/L on average, respectively. The average values of pH, ALK and Hardness in source water were 6.92, 28.6 mg/L and 27.8 mg/L, respectively, and they were slightly higher in finished water. Fluoride concentration was significantly increased from 0.10 mg/L in source water to 0.92 in finished water due to fluoridation treatment. The one-way ANOVA analysis showed that the concentrations of all parameters were significantly

different between finished water samples and source water samples except hardness (The significant level was set at 0.05).

6.3.2 Microbial recovery

The results for both filters indicate effective recovery of all the tested microbes (Table 6.3). In source water, the average recoveries of the F80A filter for *E. coli* KO11, *E. coli* O157:H7, bacteriophage MS2, *Bacillus atrophaeus* spores and adenovirus 41 were 93%, 57%, 106%, 77% and 41%, respectively. In finished water, the average recoveries of the F80A filter for *E. coli* KO11, *E. coli* O157:H7, MS2, *Bacillus atrophaeus* spores and adenovirus 41 were 74%,77%, 112%, 23% and 61%, respectively. The results of one-way ANONA indicated that recoveries for different test microbes were statistically significant ($p<0.01$) in source water samples as well as in finished water samples ($p\leq0.05$). Besides the F80A filter, the F200A filter was also tested to recover these five test microbes in both finished water samples and source water samples. The results of statistical analysis showed that the recoveries of both types of filters were not significantly different for each test microbes in both source water samples and finished water samples ($p>0.05$).

One-way ANOVA was also used to compare the recoveries of each test microbe in source water sample with that in finished water samples. The recoveries for *E. coli* KO11, *E. coli* O157:H7, MS2 in source water samples and finished water samples had no significant difference ($p>0.05$). However, the recovery for *Bacillus atropeaus spores* in source water samples (82%) was significantly higher than that in finished water samples (25%) ($p<0.01$), and the recovery for adenovirus 41 in source water samples (33%) was significantly lower than that in finished water samples (65%) ($p=0.03$).

Two filtration setups were applied to recover multiple classes of microbes in seeded test water. As demonstrated in Figure 6.2, the recoveries obtained by both filter setups were very similar in finished water as well as in source water. In terms of the specific microbes, the microbe recoveries from the normal setup model for *E. coli* KO11, *E. coli* O157:H7, MS2, *Bacillus atrophaeus* spores and adenovirus 41 were 87%,72%, 116%, 52% and 59%, respectively. For the parallel setup model, the microbe recoveries for *E. coli* KO11, *E. coli* O157:H7, MS2, *Bacillus atrophaeus* spores and adenovirus 41 were 98%, 93%, 102%, 62% and 35%, respectively. The recoveries obtained by two models were not significantly different ($p>0.05$).

The effects of 5 factors (water type, filter type, filtration setup, microorganism type and seeding levels) on the recovery were analyzed by the multi-way ANOVA. For the main effects, only the microorganism type influenced the recovery significantly ($p=0.037$ (Table 6.4). Interaction effects were found between microorganism type and water type ($p=0.021$), indicating that the recovery efficiencies of two microorganisms, *Bacillus atropeaus spores* and adenovirus 41, were significantly different between source water and finished water.

6.3.3 Influences of water pH

The microbial recovery results for both finished water samples and source water samples at pH 6.5, 7.5 and 8.5 are shown in Figure 6.5. The results of statistical analysis by One-way ANONA showed that the recovery of each test microbe was not significantly affected by water pH at 6.5 to 8.5 ($p>0.05$). In source water samples, the recovery of adenovirus 41 gradually increased as water pH was raised from 6.5 to 8.5, but the difference was not statistically significant ($p=0.10$).

6.3.4 PCR inhibition and control

PCR inhibition was determined by comparison of adenovirus concentration in the external standard with that in finished water and source water samples. The results showed that the observed concentrations of adenovirus 41 were much lower that the expected concentration in source water samples but slightly higher than expected concentrations in finished water samples, however, differences were not statistically significant ($p>0.05$). The result suggested that PCR inhibitors might exist in some source water samples but not in finished water samples (Table 6.6). When samples were subjected to chloroform extract, the observed concentrations and the expected concentrations were significantly different in finished water samples ($p=0.02$) but not in source water samples ($p=0.55$). The result suggested that chloroform extraction removed a fraction of virus in water samples and might also remove PCR inhibitors in source water.

6.3.5 Filtration processing times for standard and parallel models and for different HFUFs

The filtration times of two types of filters are listed in Table 6.7. For the normal model, the average time process 10 L of water samples was 22 minutes. In contrast, the parallel model only took 13 minutes to filter 10 L of water samples, which is significant less than that of the normal model ($p<0.01$). The F80A filter was significantly faster than the F200A filter to process 10 L of water either in the normal model ($p=0.02$) or in the parallel model ($p<0.01$). However, the processing times for 10 L of source water samples were not significant different from that for 10 L of finished water in both the normal model ($p=0.21$) and the parallel model ($p=0.94$).

6.4 Discussion

Ultrafiltration is an emerging technique to concentrate microbes in water. It is proven that hollow fiber ultrafilters are capable of concentrating viruses, bacteria, and protozoa simultaneously in large volume water samples (Morales-Morales et al., 2003). However, recovery efficiencies of HFUF are variable in different studies and within the same study. This present study provides a comprehensive evaluation of the performance of hollow fiber ultrafilters by analysis of multiple influencing factors, including diverse microbes, water types, two different filters, and two filtration setups.

Our results revealed that HFUF could recover and concentrate all of the classes of microbes tested, but with different recovery efficacy. *E. coli* KO11 and *E. coli* O157:H7 had similar recoveries, which ranged from 75% to 104%. The recovery of MS2, a viral indicator, was higher ($p=0.03$) than that for *E. coli* bacteria, ranging from 98% to 130%. The average recovery of *Bacillus atrophaeus* spore was about 56%, and recovery for adenovirus 41 was 48%, which is the lowest of all microbes tested but not significantly different from that of *Bacillus atrophaeus* spore.

The recovery differences among different test microbes by hollow fiber ultrafilters might be associated with the characteristics of microbes. These 5 microbes are much different in surface properties, morphology, and the size. *E. coli* is a Gram-negative, facultative anaerobic and non-sporulating bacterium with a typically rod-shaped cell about 2 μm long and 0.5 μm in diameter. Bacteriophage MS2 is bacteriophage with an icohahedral capsid composed of ? protein subunits and diameter of 27-34nm. Adenovirus 41, a non-enveloped DNA virus of the *Adenoviridae* family, with an icosahedral capsid structure, fibrous spikes protruding from the capsid vertices and a diameter of about 70 to 90 nm. It

is possible that these physico-chemical differences affected the retention and adsorption of the test microbes by the hollow fibers during the filtration process.

In previous studies, it is suggested that the physical and chemical quality of the water matrix could significantly affect the recovery of microbes. Sobsey and Glass (1984) reported that soluble organic compounds in water reduced the recovery efficiencies of electropositive (Virosorb lMDS) filters for enteric viruses. A study conducted by Simmons (2007) indicated that recoveries for multiple microbes by HFUF were much different between drinking water from San Francisco, California and Chapel Hill, North Carolina. Lambertini et al. (2008) found that the pH value of water samples significantly affected recovery efficiency for viruses by glass wool filters. In the present study, water quality substantially influenced the recoveries for *Bacillus atrophaeus* spores and adenovirus 41. Specifically, the recovery for *Bacillus atropeaus spores* in source water samples (82%) was significantly higher than that in finished water samples (25%) ($p<0.01$), and the recovery for adenovirus 41 in source water samples (33%) was significantly lower than that in finished water samples (65%) ($p=0.03$). Both test waters were significantly different in many physical and chemical quality parameters, especially in turbidity, Mn, and TOC and DOC.

Unlike electropositive filters and glass wool filters, hollow fiber filters are less affected by water pH when used to concentrate microbes. Our results showed that test water pH values from 6.5 to 8.5 did not affect the recoveries for multiple microbes. This suggests that hollow fiber filters could be widely applied for concentrating microbes from waters with different pH levels.

The characteristics of filters, including their chemical composition, membrane surface area, pore size and hollow fiber shape might also influence their recovery of microbes. Two types of filters Hemoflow F80A and Optiflux F200A were tested for microbe recovery. Both filters are made of the same material (polysulfone) and have similar pore size. The commercial prices of both filters are similay, about 25 $USD per unit. However, The F80A filter contains hollow fibers with a conventional shape and 1.8 m^2 of membrane surface area, while the F200A filter contains hollow fibers with a wavy shape and 2.0 m^2 of membrane surface area. The recovery results achieved by F200A were higher than those by the F200A filter, the difference was not significant. It is possible that the difference in hollow fiber shape leads to different microbe flow patterns or their opportunities to encounter the wall surfaces of the filter. Perhaps microbes are subjected to more barriers in F200A filters than in F80A, thus resulting in greater microbe retention in the F200A hollow fibers.

A variety of microbe seeding levels were tested in these experiments. The multi-way ANOVA analysis suggested that seeding levels had no significant impact on microbial recoveries. This finding is consistent with the findings of others. Kuhn and Oshima (2002) reported that the recovery of C*ryptosporidiu*m oocyst in source water samples by hollow fiber ultrafilters was not significantly impacted by the seeding densities (10-10^5 oocysts).. Lambertini et al. (2008) also found that the seeding levels (from 8.5 to 2.7 x 10^7 genomic copies per liter) did not affect the recovery rates of of multiple viruses by glass wool filters. To enumerate and detect microorganism directly and conveniently, a higher level of seeding was chosen, which is common in other studies (Anderson 2008; Hill et al., 2005; Morales-Morales et al., 2003). In practice, concentrations of pathogens in

contaminated water are normally lower than the concentrations spiked into water in these spiked sample experiments.

Time-consuming is concerned when hollow-fiber ultrafilters are used to filter a large volume of water. Commonly used disposable hollow-fiber filters have small diameter filter housing inlet and outlet openings that restrict flow, causing it to take approximate 60 minutes to filter 10L of water (Anderson 2008). In this study, the hollow-fiber ultrafilters were fitted with modified endcaps having larger inlet and outlet diameters. (Figure 6.1) This modification increased sample flow rate and decreased processing time while maintaining method recovery efficiency (Anderson 2008). The normal setup model only takes 20-25 minutes to filter 10 L water sample. To make the filtration process faster, a filter setup with two HFUFs operated in parallel was evaluated. The results indicated that both filter setups had similar efficiency to recover microbial indicators and pathogens. However, the parallel model processed samples faster than normal model, taking 11-16 minutes to filter 10L of water in the parallel setup compared to taking 20-25 minutes in the normal setup. While a reduction of 10 minutes might not seem to be such a time-saving improvement for a 10-L sample, when a large volume of100L or even more is processed, the processing time advantage of the parallel model will be more clearly demonstrated. However, the parallel model uses two filters, which costs more than the normal mode using only one filter. The trade-offs between two setups need to be considered in practice.

This study demonstrated the application of hollow fiber ultrafiltration to concencentration multiple classes of microorganisms in water and what factors influence the recovery of this concentration methods. The results indicated that the types of test microbes and water

matrices might affect the recovery of this method. Other factors, such as the type of filters, filter setup, water pH and seeding levels had no impacts on the recovery. The results suggest that the hollow fiber ultrafiltration with modified endcaps can rapidly and efficiently concentrate multiple types of microbes in a large volume of water samples. However, in contrast to the negatively charged membrane filtration developed in the previous chapter, this method is relatively expensive and difficult for field application due to the inconvenience of equipment setup. Since each concentration method has its advantages and disadvantages, the selection of appropriate concentration methods is important for the detection of pathogens in water, which is mainly dependent on the objectives and the budget of the study.

Table 6.1 The microbial recovery efficiencies of HFUF in previous studies

Microorganisms	Recovery	Water type	Seeding levels	Water volume	Reference
Cryptosporidium parvum	48%	Surface	550-210000 oocyst	2L	Kuhn and Oshima, 2001
Cryptosporidium parvum	87.7%	Drinking	105-156 oocyst	10L	Kuhn and Oshima 2002
	75%	Surface	12-127000 oocyst		
Cryptosporidium parvum	42%	Surface	100-150 oocyst	10L	Simmons et al., 2001
E. coli XL1-blue	95%	Surface	9×10^5 cfu	10L	Morales-Morales et al., 2003
T1 phage	73%	Surface	1×10^5 pfu		
PP7 phage	62%	Surface	1×10^6 pfu		
E. coli XL1-Blue	96%	Ground	2×10^7 cfu		
E. coli	89%	Surface	9×10^6 cfu		
E. coli XL1-Blue	92%	Surface	24000 cfu		
Coliphage MS2	34%	Drinking	10^6 pfu	10L	Hill et al., 2005
Enterococcus faecalis	80%	Drinking	10^6 cfu		
Salmonella enterica	67%	Drinking	10^6 cfu		
Bacillus globigii	33%	Drinking	10^6 cfu		
Cryptosporidium parvum	45%	Drinking	10^6 oocyst		
Echovirus 1	69%	Drinking	10^6 pfu		
Bacteriophage PP7	70%	Ground	10^7-10^8 pfu	100L	Olszewski, et al., 2005
	86%	Surface	10^7-10^8 pfu		
Bacteriophage T1	71%	Ground	10^7-10^8 pfu		
	70%	Surface	10^7-10^8 pfu		
Poliovirus type 2	82%	Ground	10^7-10^8 pfu		
	69%	Surface	10^7-10^8 pfu		
Bacteriophage Φx174	83%	Drinking	92000 pfu		
Bacteriophage MS 2	120%	Drinking	72000 pfu		
E. faecalis	120%	Drinking	8400 cfu	100L	Hill et al., 2007
C. perfringens	110%	Drinking	10000 cfu		
Cryptosporidium parvum	88%	Drinking	590000 occyst		

Table 6.2 Physical and chemical quality of finished water samples and source water samples

Water quality parameters	Source water(n=8)		Finished water(n=6)	
	Mean	Standard deviation	Mean	Standard deviation
Turbidity (NTU)	3.13	0.58	0.08	0.015
pH	6.92	0.07	8.18	0.076
ALK(mg/L)	28.6	2.0	35.8	3.97
Hardness	27.8	3.8	29.8	1.47
TOC(mg/L)	7.14	0.38	2.42	0.272
DOC(mg/L)	4.87	2.33	2.34	0.294
Mn (mg/L)	0.157	0.074	0.0036	0.0012
Iron (mg/L)	0.46	0.25	0.005	0.005
Fluoride(mg/L)	0.10	0.01	0.92	0.14

Table 6.3 Average recovery and its standard deviation (%) of microbes with different filters in source water and finished water

Water Type	Filter Type	No. trial	*E. coli* KO11	*E. coli* O157:H7	MS2	*Bacillus atropeaus* spores	Adenovirus 41	p value[a]
Source water	F80A	7	93±26[b]	67±42	106±31	77± 17	42±31	0.01
	F200A	5-7	104±14	92±21	98±25	87±32	24± 29	0.00
Finished water	F80A	6-8	74 ± 25	77± 34	112± 27	23± 14	61± 33	0.00
	F200A	4	104± 39	104 ± 65	130± 39	28±6	73± 54	0.05

a: The difference of the recovery of test microbes was tested by one-way ANOVA. For each type of filters and both source water and finished water, the recoveries were statistically different among test microbes ($p \leq 0.05$).
b: The results were presented as mean± standard deviation.

Table 6.4 Multi-way analysis of influencing factor on the test microbe recovery efficiencies by HFUF

Source	Type III Sum of Squares	df	Mean Square	F value	p
Corrected Model	16.003	72	0.222	2.582	0.000
Intercept	40.956	1	40.956	475.865	0.000
Water type	0.041	1	0.041	0.472	0.495
Filter type	0.437	1	0.437	5.083	0.028
Filtration model	1.9×10^{-8}	1	1.9×10^{-8}	.000	1.000
Microbes	1.719	4	0.430	4.993	0.002
Log seeding level	1.026	5	0.205	2.383	0.051
Water type + filter type	0.405	1	0.405	4.711	0.035
Water type + microbe type	1.678	4	0.420	4.875	0.002
Error	4.389	51	0.086		
Total	95.814	124			
Corrected Total	20.392	123			

The results of the interaction effects were omitted, which were not significant.
R Squared = 0.785 (Adjusted R Squared = 0.481).

Table 6.5 Effects of water pH on test microbe recovery efficiencies (%) by HFUF

Water type	Test microbes	Recovery (%) (mean± standard deviation)			No. trial	p value[a]
		pH=6.5	pH=7.5	pH=8.5		
Source water	*E. coli* KO11	79±14	64±0	69±1	4	0.49
	E. coli O157:H7	65±43	118±32	88±14	4	0.33
	MS2	114±40	158±20	108±22	4	0.32
	Bacillus atropeaus spores	77±14	94±12	91±11	4	0.22
	Adenovirus 41	43±25	99±27	139±84	4	0.10
Finished water	*E. coli* KO11	71±11	78±34	79±33	4	0.93
	E. coli O157:H7	89±22	72±22	75±15	4	0.55
	MS2	132±12	126±16	168±9	4	0.01
	Bacillus atropeaus spores	34±14	26±17	37±17	4	0.68
	Adenovirus 41	81±42	80±36	61±20	4	0.71

a: The difference of the recovery of test microbes at different water pH was tested by one-way ANOVA.

Table 6.6 The effects of PCR inhibition on the detection of adenovirus 41

Water type	Chloroform extract	Expected concentration (pfu/mL)	Observed concentration (pfu/mL)	Observed /expected	p value[a]
Finished water	No	12125	17000	140%	0.21
	No	12125	14500	120%	
	Yes	12125	4750	35%	0.02
	Yes	12125	4250	39%	
Source water	No	6946	3028	44%	0.12
	No	6946	1098	16%	
	Yes	6946	7068	102%	0.55
	Yes	6946	5228	75%	

a: The statistical difference between the expected concentration and observed concentration of adenovirus 41 was tested by paired-samples t test

Table 6.7 The filtration processing time of HFUF in the different setups

Water	Filter	Normal model		Parallel model		No. trial	p value[a]
		Time (min)	Flow rate (L/min)	Time (min)	Flow rate (L/min)		
Finished water	F80A	19±3	0.53	11±2	0.91	4-5	<0.01
	F200A	25±4	0.40	17±1	0.59	2	0.18
Source water	F80A	22±4	0.45	11±3	0.91	4	<0.01
	F200A	25±3	0.40	16±2	0.63	3	0.01
Average	F80A	20±3	0.5	11±2	0.91	8-9	<0.01
	F200A	25±3	0.4	16+1	0.63	5	<0.01
Average of total trials		22±4	0.45	13±3 m	0.77	13-14	<0.01

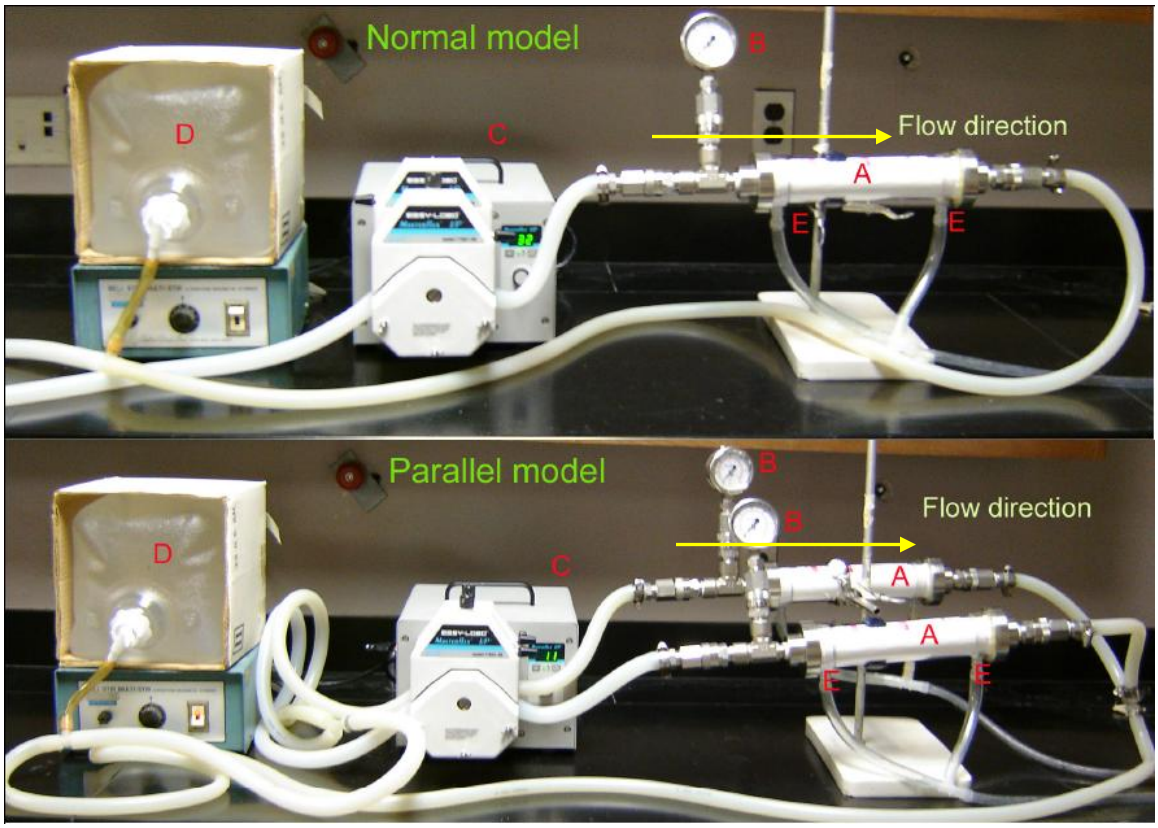


Figure 6.1 The setup of hollow fiber ultrafiltration in normal model (upper) and parallel model (down). A: Hollow fiber ultrafilter; B: gauge; C: pump; D: water sample; E: outlets of permeate

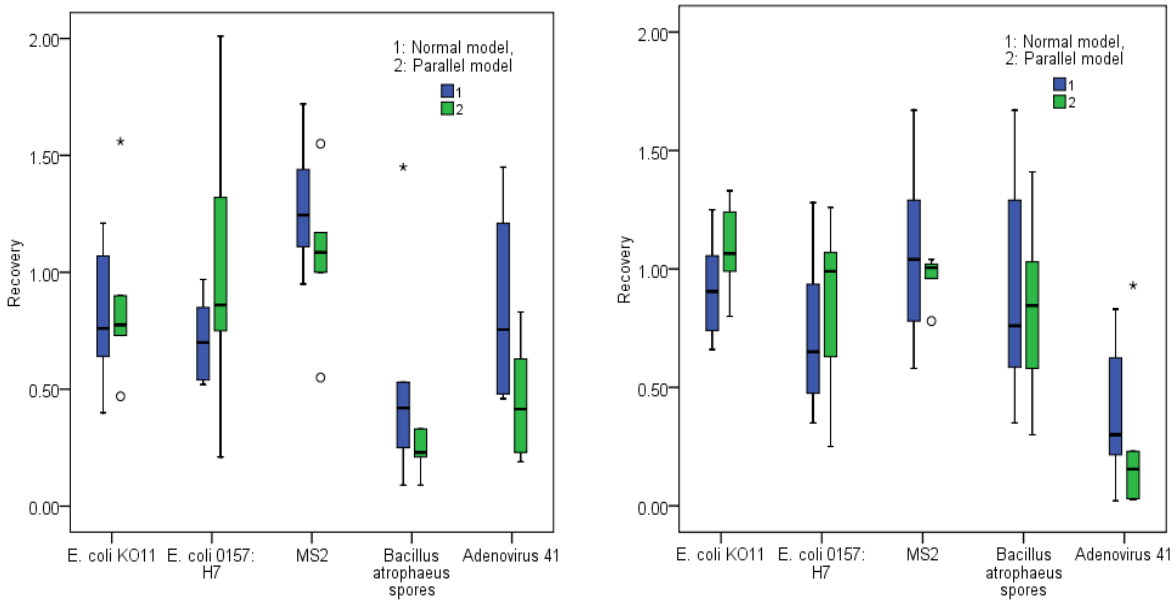


Figure 6.2 The comparison of test microbe recovery by both filter setup models in finished water (left panel, No. trial =6) and source water (right panel, No. trial=8)

Chapter 7

CONCLUSIONS AND FUTURE RESEARCH

7.1 Conclusions

The major conclusions from the papers included in this dissertation are listed below:

1). GIS and spatial statistics were applied to examine whether diarrheal diseases were clustered in space and time. All three types of diarrheal diseases were clustered either in space or space and time.

2). Tubewell density was inversely associated with childhood diarrheal diseases, namely, children are less likely to contract diarrhea if there are more tubewells around the *bari* where they live. In addition, intermediate depth wells were associated with a higher risk of childhood diarrhea than shallow wells and deep wells.

3). There was an increase in the likelihood of childhood diarrhea for children drinking from shallow wells with higher arsenic compared to wells with lower arsenic after adjusting for flood control, population density and SES. This suggests that the health benefits of reducing As exposure by switching from a shallow high-As tubewell to a shallow low-As are to some extent countered by an increase in childhood diarrhea risk.

4). The virus concentration method developed in this study is simple and cost-effective, and can be used efficiently to recover adenovirus 41 from turbid water samples. In addition, this is the first application of sample evaporation to concentrate viruses in water samples.

5). Hollow fiber ultrafiltration is an effective technique for recovering and concentrating multiple classes of microorganisms rapidly and simultaneously. However, the recovery efficiencies varied among the different microbes and different water matrices.

In summary, the main part of this dissertation involved the analysis of 60,000 cases of diarrheal disease in children under five in a population drinking primarily untreated groundwater. To my knowledge, this is the first epidemiological study focused on the relationship between childhood diarrhea, tubewell access and depth, and As levels in groundwater. The main finding shows that children under five drinking water from shallow wells that are low in arsenic are in fact more likely to have diarrheal disease. This information is important for relevant policy-makers to make appropriate strategies for waterborne diarrhea disease reduction and arsenic mitigation.

7.2 Future research

In these studies, risk factors for childhood diarrhea were examined. However, it is unknown what factors are related to diarrheal diseases caused by specific pathogens. Given the availability of data, exploring the relationship between diarrhea caused by *Shigella* and rotavirus and these risk factors related to water and other exposure routes is an important next step.

It is surprising to find that intermediate depth wells are associated with a higher risk of diarrhea compared to shallow wells. This finding merits further research. It would be interesting to evaluate whether intermediate depth wells are more vulnerable to fecal contamination. Also, mechanisms for pathogen transport into the drinking water from intermediate depth wells need to be investigated.

It was reported that groundwater in Bangladesh is prone to fecal contamination. It is still unknown whether a correlation exists between diarrheal diseases and fecal indicators or

pathogens in tubewell water. *E. coli* in tubewell water has been monitored each month since 2008 (van Geen et al., 2011) and childhood diarrhea cases are continuously collected by ICDDR, B CHWs. Given the availability of *E. coli* and childhood diarrhea data, future research should statistically evaluate whether there is an association between the concentration of *E. coli* or of other enteric microbes in groundwater and cases of diarrhea among children who drink that water in space and time.

A novel method was developed to concentrate adenovirus 41 in small volumes of source water. Additional research is needed to evaluate the efficacy of this method for concentrating other types of enteric viruses. Performance of the method in field application also needs to be demonstrated, especially in water for which there is evidence of fecal contamination and waterborne diarrheal disease risk.

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